CORTICOTROPIN-RELEASING HORMONE

AD HERMUS

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PROEFSCHRIFT
TER VERKRIJGING VAN DE GRAAD VAN DOCTOR IN DE GENEESKUNDE AAN DE KATHOLIEKE UNIVERSITEIT TE NIJMEGEN, OP GEZAG VAN DE RECTOR MAGNIFICUS PROF. DR.J.H.G.I.GIESBERS VOLGENS BESLUIT VAN HET COLLEGE VAN DEKANEN IN HET OPENBAAR TE VERDEDIGEN OP DONDERDAG 17 APRIL 1986 DES NAMIDDAGS TE 16.00 UUR.

door
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geboren te Breda

1986
Druk: ICG Printing BV, Dordrecht

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Contents

Preamble

The hypothalamic hormones TRH, LHRH and somatostatin, isolated in the late sixties and early seventies, are very useful in many clinical conditions. In 1981 the structure of a fourth hypothalamic peptide hormone, corticotropin-releasing hormone (1) was elucidated from ovine hypothalami by Vale et al (2), followed by the characterization of the structure of human CRH by Shibahara et al (3) in 1983.

In this thesis the hormonal and hemodynamic effects of the intravenous administration of ovine and human CRH in man are studied. When we started our CRH programme in May 1982 only one brief report had been published on the effects of ovine CRH administration in man (4). Therefore we decided to investigate first, which factors determine the response of the pituitary to CRH administration in healthy subjects. In the second half of this thesis the ACTH and cortisol responses to CRH in patients with hypo- and hypercorticism, both before and after treatment are reported. The outcome of our studies lead to the conclusion that CRH provides not only a new tool in the study of the physiology and pathophysiology of the hypothalamic-pituitary-adrenal axis, but is also an aid in the clinical work-up of the patient with hypo- or hypercorticism.

REFERENCES AND NOTES

1. The name corticotropin-releasing *factor* (CRF) was replaced by corticotropin-releasing *hormone* (CRH) in a number of journals during the course of this study. Therefore in some chapters of this thesis the name corticotropin-releasing factor was used and in others corticotropin-releasing hormone. Furthermore, in the British journal Clinical Endocrinology the name corticotrophin-releasing factor had to be used.
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Chapter 1

Review of animal studies

Review of animal studies

1.1 ISOLATION AND CHARACTERIZATION OF CRF

Fifty years ago Harris (1) was the first to suggest that the secretion of hormones by the anterior pituitary was governed by humoral factors from the hypothalamus. In 1950 De Groot and Harris (2) showed that electrical stimulation of the hypothalamus led to a remarkable increase in the activity of the adrenal cortex. They postulated that the hypothalamus released a humoral factor into the hypothalamo-hypophyseal portal vessels, which stimulated ACTH release by the pituitary. In 1955 Saffran and Schally (3) and Guillemin and Rosenberg (4) independently demonstrated the presence of such a factor, which they called corticotropin-releasing factor (CRF).

It was not until 1981, however, that Wylie Vale and co-workers from the Salk Institute in La Jolla isolated from 490.000 ovine hypothalami, earlier used for the isolation of TRH and LHRH, a polypeptide of 41 amino acids (molecular weight 4671) which fulfilled the criteria of a corticotropin-releasing factor (5,6). The biological activity of the molecule was found to depend on the integrity of the C-terminal 27 amino acids (5,7). The isolation of ovine CRF (oCRF) was followed by the characterization of rat CRF (rCRF, molecular weight 4758) in 1983 by Rivier et al (8,9) and the elucidation of the structure of human CRF by analysis of the human pre-pro CRF gene by Shibahara et al (10). Both appeared to be peptides of 41 amino acids with identical amino acid sequences, differing from ovine CRF in 7 amino acid residues. Recently the structures of bovine (11) and caprine CRF (12) were elucidated. CRF has a 50% homology with two non-mammalian CRF-like peptides, namely sauvagine, a polypeptide of 40 amino acids, isolated from the skin of the South American frog Phylomedusa sauvagei (13) and Urotensin I, a polypeptide, isolated from the urophysis of two species of fish, Cyprinus carpio and Catostomus commersoni (14,15). CRF

is derived from a precursor molecule. The structure of this precursor (pre-pro CRF) has been elucidated in sheep (190 amino acids) (16), rat (187 amino acids) (17) and man (196 amino acids) (10).

1.2 DISTRIBUTION OF CRF IN THE BRAIN

Like most other neuropeptides, CRF is widely distributed throughout the brain (18-28). Using antisera directed against ovine or rat CRF, major concentrations of CRF immunoreactive neurons in the rat brain have been described in:

1. the hypothalamus, especially in the paraventricular nucleus. In the colchicine treated rat approximately 2000 of the 10.000 perikarya in this nucleus contain CRF (18). 80% of the CRF positive cell bodies are localized in the parvocellular division and 15% in the magnocellular division of this nucleus. The great majority of CRF neurons in the paraventricular nucleus projects to the external layer of the median eminence. This system is responsible for regulating the release of proopiolipomelanocortin (POMC) derived peptides from the pituitary. A minority of CRF neurons from the hypothalamus projects to the neurointermediate lobe of the hypophysis (29,30).
   Fibres from other parts of the hypothalamus, from the nucleus tractus solitarius, from the subfornical organ and the limbic system supply neural input to the parvocellular division of the paraventricular nucleus (18).
2. parts of the limbic system and several nuclei of the basal forebrain and brain stem, which are known to be involved in the mediation of a variety of autonomic responses to stress.
3. the cerebral cortex.

Studies of CRF immunoreactivity in various regions of the brain using radioimmunoassays provided data fairly consistent with immunohistochemical findings (26,27,31,32). In rats and in sheep the highest concentrations of CRF-like immunoreactivity were found in the median eminence, whereas the concentrations in other brain regions were at least two orders of magnitude lower (26,27,32). High affinity CRF receptors have been demonstrated in the anterior and intermediate pituitary, the external layer of the median eminence, parts of the limbic system and in the forebrain (33-35).

After bilateral adrenalectomy CRF immunostaining in cell bodies of the paraventricular nucleus is enhanced, whereas CRF immunoreactivity in the median eminence is decreased, suggesting a high state of activity of CRF containing neurons (36). Recently Jingami et al (37) found that adrenalectomy increased hypothalamic pre-pro CRF mRNA content. Moldow and Fishman (38) showed that treatment with dexamethasone reduced CRF-like immunoreactivity of the rat hypothalamus two fold, while Suda et al (39) demonstrated the same for the median eminence. These observations

illustrate that glucocorticoids can exert a negative feedback effect on the hypothalamus.

A number of studies have demonstrated the colocalization of other neuroactive substances in individual parvocellular CRF neurons (40-48). In the colchicine treated rat there is extensive colocalization of CRF with metenkephalin and peptide histidine isoleucine (PHI) immunoreactivity (40), whereas a smaller subset of CRF neurons contain both CRF and neurotensin (41) or CRF and oxytocin or vasopressin (41). Colocalization of CRF and vasopressin immunoactivity is enhanced after adrenalectomy (42-45), whereas in the adrenalectomized, colchicine treated rat individual neurons contain both CRF and dynorphin (47) or angiotensin II (48).

1.3 ACTION OF CRF ON THE ANTERIOR LOBE

Ovine and rat CRF exhibit high potency and intrinsic activity to stimulate release of ACTH and β-endorphin in vitro (5,8,49-53) as well as in vivo (54-57). oCRF and rCRF have similar potencies in stimulating ACTH release from cultured rat pituitary cells (minimal effective concentration $1\text{-}10\times10^{-12}$M, ED_{50}value $20\text{-}200\times10^{-12}$M, plateau response $1\text{-}10\times10^{-9}$M) (5,8,53). In freely moving rats (54) and in stalk-sectioned cynomolgus monkeys (55) bolus injections of oCRF with doses as low as 0,5 μg/kg produce a significant activation of the pituitary-adrenal axis. In sheep (56,57) equivalent doses of oCRF stimulate ACTH and β-endorphin secretion. CRF not only stimulates the *release* of ACTH and the other POMC derived peptides from the anterior pituitary, but also promotes their *synthesis,* as derived from the increase of POMC mRNA levels after oCRF administration (58,59). The effect of CRF on ACTH secretion is mediated via Ca dependent (60,61) generation of cAMP (7,62-64). After binding to specific receptors (33) located in the plasma membrane of corticotrophs, CRF is rapidly internalized and migrates to the Golgi apparatus and lysosomes, where degradation may occur (65,66).

In rats ovine CRF specifically stimulates the release of POMC fragments in the anterior pituitary, whereas in cynomolgus and rhesus monkeys (55,67) the peptide also causes dose-dependent prolactin and growth hormone responses. After intravenous administration of oCRF to rats not only an increase of corticosterone, but also of aldosterone and its precursor 18-hydroxycorticosterone has been described (68,69).

It has been shown that dexamethasone modulates the response of cultured rat anterior pituitary cells to ovine CRF by a non-competitive interaction (53). The inhibitory effect of dexamethasone on the response to CRF is dose-dependent as well as time-dependent (53). Maximum inhibition of CRF induced ACTH release was observed after 4 hours of preincubation with dexamethasone. Bilezikjian et al (70) and Shimizu et al (71) showed that the

inhibitory influence of dexamethasone on CRF induced ACTH secretion in vitro is mediated, at least in part, by a negative effect on cyclic AMP formation. However, a site of action distal to cyclic AMP production has been claimed by Giguère et al (64).

In vivo, Rivier et al (54) showed that pretreatment with 20 μg dexamethasone intraperitoneally 4 hours before ovine CRF administration almost completely abolished the ACTH response to CRF in doses of up to 3 μg in anaesthetized rats. A 95% decrease in ACTH response to 10 μg ovine CRF after pretreatment with 100 μg dexamethasone 15 and 3 hours before CRF injection in rats has been shown by Proulx-Ferland et al (72). In sheep, Donald et al (73) demonstrated that 0,4 - 4 mg dexamethasone, intravenously administered 2 hours before injection of 200 μg ovine CRF, abolished ACTH and cortisol responsiveness.

It has been demonstrated that repeated injections or a continuous infusion (24 hr - 7 days) of oCRF in rats causes some decrease of the pituitary response to CRF (74,75). However, this loss of response is small when compared to the degree of pituitary desensitization observed during repeated exposure to other releasing factors, e.g. GnRH. This decrement of the response to CRF is in part caused by increased negative feedback, due to elevation of glucocorticoid levels.

1.4 ACTION OF CRF ON THE INTERMEDIATE LOBE

Peptides, derived from the precursor molecule proopiolipomelanocortin (POMC) are produced in the anterior as well as in the intermediate lobe of the pituitary. However, the processing of POMC is different in both hypophyseal compartments. ACTH, β-endorphin and β-lipotropin are the main products of the pars anterior, whereas in the pars intermedia ACTH is further cleaved in α-MSH and CLIP (corticotropin like intermediate peptide) (76).

The rate of secretion of POMC derived peptides in rat pars intermedia cells results from a balance between the stimulatory effects of β-adrenergic agents (epinephrine) and the inhibitory influence of dopamine. Recently, however, it has been demonstrated that in addition to β-adrenergic agents CRF also has a stimulatory effect on the rat intermediate pituitary (53,72,77,78).

Meunier et al (77) showed that oCRF stimulates α-MSH release in rat pars intermedia cells in culture. The effect of CRF on α-MSH release in these cells is observed at an ED_{50} value of 10^{-9}M and is mediated by an increase of adenylate cyclase activity (78). Whereas the dopaminergic agonist bromocriptine inhibits the CRF induced α-MSH release, dexamethasone pretreatment, under conditions which lead to an almost complete inhibition of ACTH secretion in corticotrophs of the anterior pituitary gland, has no

inhibitory effect on either spontaneous or CRF induced α-MSH secretion in vitro (77).

In vivo Proulx-Ferland et al (72) showed that in freely moving rats intravenous administration of 10 μg oCRF not only leads to a sixfold increase of the plasma concentration of ACTH, but also to a similar stimulatory effect on plasma α-MSH. Moreover, these authors demonstrated that administration of 100 μg of dexamethasone 15 and 3 hours before the injection of CRF led to a 95% decrease in the ACTH response to oCRF, while the α-MSH response remained unchanged. Vale et al (53), studying β-endorphin release from rat intermediate lobe cells in culture, demonstrated that the sensitivity of intermediate lobe cells for CRF is at least one order of magnitude less than that of adenohypophyseal cells and that maximally effective CRF concentrations stimulated β-endorphin secretion in rat intermediate lobe cells to a lesser degree than did epinephrine. Gibbs et al (79) found that oCRF had no effect on β-endorphin, β-lipotropin or α-MSH secretion from neurointermediate lobes of human fetuses, regardless of age.

From this data one might infer that CRF, at least in the rat, can interact with dopamine in the physiological control of the intermediate lobe, although, based on the above mentioned and other data (80,81), epinephrine seems to be more important than CRF as a regulator of intermediate lobe function.

1.5 INTERACTIONS OF CRF WITH OTHER HYPOTHALAMIC FACTORS IN THE CONTROL OF ACTH RELEASE

The rat hypothalamus and portal blood contain a number of other factors in addition to CRF, capable of inducing ACTH release from cultured anterior lobe cells. This paragraph summarizes some recent studies on the ACTH releasing and CRF potentiating properties of these factors, which have a lower potency and intrinsic activity than CRF for the release of ACTH (53).

The ACTH releasing capacity of vasopressin, known for many years, is illustrated by the observation that in vitro preincubation of stalk median eminence extracts of Wistar rats with an antibody to vasopressin reduces its ACTH releasing ability by 60% (82). The role of vasopressin in ACTH secretion is probably complex, since apart from increasing pituitary ACTH secretion (53,83), it has also been reported to cause the release of endogenous CRF (84,85) and to potentiate the action of this peptide on the anterior lobe (53,83,86-89). However, a facilitatory action of vasopressin on the release of CRF has been questioned by others (90). The action of vasopressin on the corticotropic cell is probably mediated by V_1 (pressor-like) receptors (91,92), although a novel (V_3= non-pressor, non-antidiuretic) type of receptor is assumed to be involved in this action by others (93,94).

Vasopressin itself acts through a cAMP independent pathway (95), although it can potentiate the CRF induced cAMP accumulation in corticotropic cells (87,95).

Interactions between CRF and other factors have also been demonstrated. The weak ACTH secretagogues angiotensin II (96,97), peptide histidine isoleucine (PHI) (98,99) and oxytocin (53,96,100,101) markedly potentiate CRF activity on the anterior lobe in vitro. Furthermore epinephrine and norepinephrine have a weak ACTH releasing activity in vitro (53) by means of an α_1-adrenergic receptor, whereas both in vitro and in vivo administration of these catecholamines enhance the effect of CRF on ACTH release (53,83,102). It was shown that somatostatin inhibits CRF induced ACTH secretion in mouse pituitary tumor cells (103,104).

Finally, it has to be stressed that immunoneutralization of endogenous CRF either markedly reduces (vasopressin) or completely abolishes (oxytocin, angiotensin II, epinephrine) the ACTH release induced by the administration of these factors in vivo (83,105,106). This illustrates the vital role for CRF in regulating ACTH release.

1.6 LEVELS OF CRF AND OTHER PUTATIVE ACTH REGULATING FACTORS IN HYPOPHYSEAL PORTAL BLOOD

Using antisera directed against ovine or rat CRF, CRF-like immunoreactivity has been demonstrated in portal plasma of anaesthetized rats in concentrations as low as 10^{-10}Mol (450 pg/ml; secretory rate 1,5 pg/min) (107-115). It has to be noted that concentrations of other hypothalamic hormones involved in the control of ACTH secretion are about 10-fold (arginine-vasopressin, oxytocin) or even 100-fold (epinephrine) higher (108-115). These portal blood levels are significantly higher (2x for epinephrine, 10x for arginine-vasopressin and 30x for oxytocin) than those in the peripheral plasma of the rat, in which no CRF immunoactivity has been detected up till now (108,113). These systemic to portal concentration gradients strongly support a central origin for each of these factors. Moreover, the hormone levels in portal plasma are well within the range of concentrations shown to evoke ACTH secretion by pituitary cells in vitro, when presented alone or in combination.

Table 1 illustrates the changes of portal blood levels of these 4 hormones in anaesthetized rats in 4 situations known to alter ACTH secretion. Surprisingly, significant changes of CRF levels were only observed after volume depletion (hemorrhage of 15% of blood volume), whereas significant changes of AVP levels occurred in all experimental conditions. However, it is thought that in situations with pituitary-adrenal activation without a rise in portal CRF levels, CRF plays at least a permissive role, as is illustrated by the fact that no ACTH rise occurs after insulin-induced hypoglycemia following

pretreatment with an antiserum to rat CRF (113,114). The significant role for CRF in mediating ACTH secretion is also illustrated by the complete abolishment of CRF and ACTH stimulation by hemorrhage stress following pretreatment with dexamethasone or by lesions of the paraventricular nucleus (113,115)

Table 1 Relative changes of peripheral ACTH levels and portal CRF, AVP, OT, and E levels to 4 different stimuli in anaesthetized rats

	Peripheral	Portal blood			
	ACTH	*CRF*	*AVP*	*OT*	*E*
Depletion of blood volume[108,113]	+300%	+140%	+110%	+110%	+230%
Insulin-induced hypoglycemia[113]	+150%	=	+ 80%	n m	n m
Hypothermia[109,112]	- 62%	=	- 35%	- 35%	n m
Expansion of blood volume[108,113]	- 70%	=	- 49%	=	=

= no significant changes
n m not measured
AVP = arginine vasopressin, OT= oxytocin, E= epinephrine

This data favour the concept that CRF is not the only corticotropin-releasing factor. It seems most likely that the hypothalamo-hypophyseal-adrenal axis can be activated in different ways in response to different stimuli. Although CRF seems to be the central hormone in the hypothalamic control of ACTH secretion, additional hypothalamic hormones, including arginine-vasopressin, oxytocin, epinephrine, and possibly other factors (112), may in some conditions play a role in the stimulation of ACTH In the next paragraph we will look, in more detail, into the respective roles of these factors in the activation of the pituitary-adrenal axis during stress

1 7 THE ROLE OF CRF AND OTHER FACTORS IN THE RELEASE OF ACTH DURING STRESS

The concept that stress induced ACTH release is not controlled by a single factor, but by the interaction of several substances, including CRF, vasopressin and catecholamines is now widely recognized. Of these ACTH releasing factors CRF has the highest potency, intrinsic activity and specificity for the stimulation of the release of ACTH and other POMC derived peptides (53). This paragraph briefly summarizes evidence of the significance of CRF, vasopressin and catecholamines respectively in the stress induced activation of the pituitary-adrenal axis

The pivotal role of CRF during stress was demonstrated by Rivier et al (116) and Rivier and Vale (117), who showed that intravenous admini-

stration of rabbit antiserum to ovine or rat CRF blocked 85% of the ACTH release observed in rats exposed to ether stress. Linton et al (118), using an antiserum to ovine CRF, observed that the rise in plasma ACTH induced by formalin stress was reduced to 28% in rats pretreated with anti CRF as compared to the response in normal rabbit serum treated animals, whereas the ACTH response to restraint stress was attenuated to 13%. Bilateral lesions of the paraventricular nucleus (PVN), leading to a 90% reduction of CRF-like immunoreactivity content of the median eminence, attenuate the ACTH response to ether stress by 70-85%, whereas when PVN lesioned rats were treated with the ganglionic blocker chlorisondamine - a blocker of peripheral catecholamine secretion - or with the vasopressin antagonist desaminopenicillamine-1 (O-methyl) tyrosine-2-aVP the ACTH response to ether stress was completely abolished (119). Moreover, pretreatment of rats with the CRF antagonist α-helical CRF_{9-41} prevented most, but not all, of the increase in ACTH caused by ether stress (120). Direct evidence of the vital role of CRF was also obtained from the observation that production of an antiserum to ovine CRF led to severe secondary adrenal failure in a rabbit (121).

Although this data strongly suggests that increased CRF secretion is the cause of the activation of the pituitary-adrenal axis during stress, a number of observations support the notion that other factors play at least a modulatory role (122).

The role of vasopressin in the control of the pituitary-adrenal response to stress is supported by the observation that in the intact rat treatment with an antiserum to vasopressin reduced the rise in plasma ACTH during formalin stress to 53% and in restraint stress to 37% (118). Rivier and Vale (117) and Bruhn et al (119) showed that the aVP antagonist desaminopenicillamine-1 (O-methyl) tyrosine -2-aVP had no effect on the early phase of the ACTH response to ether stress, but reduced this response by 45% in later (10-20 min) phases of ether stress. However, it is most likely that, in addition to CRF and vasopressin, at least one other factor is relevant for the ACTH response during stress, since Linton et al (118) found that treatment with anti CRF together with anti vasopressin could not entirely prevent the ACTH response in the rat during formalin and restraint stress.

Rivier and Vale (117) claim that circulating catecholamines play a role in the activation of the anterior pituitary during stress. They demonstrated that treatment with chlorisondamine inhibits ACTH release in rats during ether stress by 40-60% and by 100% when it is administered together with anti CRF. However, other workers (122,123) found no evidence that circulating catecholamines are of significance in the pituitary-adrenal response to stress, although circulating epinephrine seems to play a major role in the release of intermediate lobe peptides during emotional stress (122,123).

Together this data favours the notion that it is highly likely that CRF is the

predominant regulator of stress induced ACTH secretion, but that vasopressin and possibly other factors (epinephrine, oxytocin, angiotensin II) are also involved in mediating stress induced ACTH secretion, most probably as CRF potentiating agents.

1.8 EXTRA-PITUITARY ACTIONS OF CRF

Stressful stimuli evoke concurrent activation of the pituitary-adrenal axis and the sympathetic nervous system and result in metabolic, cardiovascular and visceral organ function changes, which can be interpreted as a generalized adaptive response to stress. Several studies (124-132) have demonstrated that CRF not only plays a key role in the hormonal response to stress, but also acts within the central nervous system to elicit a variety of effects, regularly observed during stress.

CRF given intracerebroventricularly (i.c.v.) to rats or dogs not only activates pituitary-adrenal function, but also elicits dose related elevations of plasma epinephrine and norepinephrine concentrations, indicating activation of the sympathetic nervous system and the adrenal medulla (124, 125, 128, 130, 131). Activation of the sympathetic nervous system by intracerebroventricular administration of CRF in the rat results in increases of plasma glucose, mean arterial pressure and heart rate (124-132). CRF induced elevations of plasma glucose, epinephrine, norepinephrine, mean arterial pressure and heart rate are prevented by administration of the ganglionic blocker chlorisondamine (125, 126, 128, 131). Intracerebroventricular administration of CRF also increases total body oxygen consumption (124), induces behavioral changes (for reviews see 133 and 134) and suppresses gastric acid secretion in rats and dogs (135-138).

Apart from controlling ACTH release by a direct effect at the pituitary level, CRF has been found to act centrally to inhibit the secretion of LH and growth hormone (83,139-141). CRF induces a dose-dependent inhibition of LH (but not of FSH) release in gonadectomized/adrenalectomized rats, indicating that this effect is not mediated through steroids of either adrenal or gonadal origin (83,139). This deleterious effect of central CRF administration does not involve opiate or peripheral catecholaminergic pathways (83,139), but is probably mediated by a direct inhibitory effect on hypothalamic LHRH release (142). Intracerebroventricular CRF administration also inhibits growth hormone secretion (140,141). It has to be determined whether this effect results from inhibiting growth hormone releasing hormone release or from stimulating somatostatin release (143). Furthermore it has been demonstrated that CRF administered i.c.v. inhibits vasopressin and oxytocin secretion into hypophysial portal blood (110). These observations strongly suggest that CRF might indeed be a key signal in mediating and integrating an organism's endocrine, visceral and beha-

vioral responses to stress

Several reports have demonstrated that CRF immunoreactivity is not restricted to the brain, but is also found in a variety of peripheral organs Of special interest is localization of CRF in the stomach and small intestine (144,145), pancreas (144,146) and adrenal medulla (147-149). The functional significance of this peripheral CRF has to be determined Paracrine effects of CRF are postulated in the pancreas (on insulin release (150)) and in the adrenal medulla (on adrenalin secretion (148))

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Chapter 2

Studies in healthy human subjects

2.1 PLASMA ADRENOCORTICOTROPIN, CORTISOL, AND ALDOSTERONE RESPONSES TO CORTICOTROPIN-RELEASING FACTOR: MODULATORY EFFECT OF BASAL CORTISOL LEVELS

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SUMMARY

Two hundred micrograms of ovine corticotropin-releasing factor (CRF) were administered as an iv bolus injection to 10 normal subjects (5 men and 5 women). Mean plasma ACTH levels rose significantly ($P < 0.0005$, by Friedman's nonparametric analysis of variance) from a basal value of 27 ± 5 pg/ml (mean $\pm$ SEM) to a peak value of 63 ± 8 pg/ml 30 min after CRF administration. This ACTH response was followed by a rise in mean plasma cortisol levels ($P < 0.0005$, by Friedman's test) from a baseline value of 12.3 ± 1.4 μg/100 ml to a peak value of 21.0 ± 0.7 μg/100 ml 60 min after CRF and a rise in mean plasma aldosterone levels from a basal value of 13 ± 2 ng/100 ml to a peak value of 23 ± 2 ng/100 ml. There was no significant difference between men and women in the responsiveness of ACTH, cortisol, and aldosterone to CRF administration. The individual basal cortisol levels were highly significantly and negatively correlated with the areas under the individual ACTH curves ($r = -0.76$; $P < 0.005$, by Pearson's correlation test) and cortisol curves ($r = -0.91$; $P < 0.001$, by Pearson's test). These data suggest a modulatory effect of physiological cortisol levels on the response of the pituitary-adrenal axis to CRF.

INTRODUCTION

Shortly after the elucidation of the structure of ovine corticotropin-releasing factor (CRF) by Vale et al. (1), several reports appeared dealing with the effects of CRF on the secretion of ACTH, cortisol, aldosterone, and various pituitary hormones in laboratory animals (2,3) and in normal humans (4-6). In patients with Cushing's disease, enhanced responsiveness of ACTH and

cortisol to CRF generally has been found (5,7,8). In our study the responsiveness of cortisol to CRF was most pronounced in those patients with Cushing's disease who had the highest basal plasma cortisol levels (8). This prompted us to study the modulating effect of basal cortisol levels on the response to CRF in normal humans. In contrast to patients with Cushing's disease, the magnitude of the cortisol response to CRF was inversely related to the basal plasma cortisol level, i.e. the higher the basal cortisol level, the lesser its response to CRF. Furthermore, CRF stimulated not only ACTH, and thereby cortisol secretion, but also the release of aldosterone.

MATERIALS AND METHODS

Ten normal subjects (five men, aged 23 ± 3 yr [mean ± SD], and five women, aged 31 ± 14 yr) received 200 μg ovine CRF (Bachem, Torrance, CA) dissolved in 2 ml acid-saline (pH 2) as a bolus injection. Informed consent was obtained from all volunteers, and the protocol was approved by the hospital ethical committee. All tests were performed at 09.00 h with the subjects fasting and at bed rest. Previous sodium intake was normal. Blood samples for hormone assay were collected at -30, 0, 1.5, 3, 5, 7.5, 10, 20, 30, 60, 120, 180 and 240 min via an indwelling iv cannula kept open with a diluted (10%) solution of heparin. The cannula was inserted 1 h before CRF administration. Plasma ACTH [intraassay coefficient of variation (CV), 12%] (9), cortisol (CV, 8%) (10), LH (CV, 7%), FSH (CV, 9%) (11), TSH (CV, 6%) (12), PRL (CV, 6%) (13), human GH (CV, 15%) (14), and aldosterone (CV, 8%) (15) were determined by specific RIAs, as described previously. Statistical analyses were performed using Wilcoxon's paired rank test (P values denoted by P), Friedman's nonparametric analysis of variance ($P = P^*$), and Pearson's correlation test ($P = P^{**}$). Unless otherwise stated, the mean values ± 1 SEM are given.

RESULTS

Side effects

Flushing, mild dyspnea, and small decreases in diastolic blood pressure occurred in most subjects immediately after CRF administration. Major complications did not occur in these normal subjects (16,17).

Responses of PRL, TSH, LH, FSH, and human GH (hGH)

Mean plasma PRL levels significantly ($P^* < 0.0005$) decreased throughout the test from 343 ± 51 mU/liter at -30 min to 255 ± 36 mU/liter at 0 min ($P <$

0.01 vs. -30 min) and 125 ± 23 mU/liter at 120 min ($P < 0.01$ vs. 0 min). Mean plasma TSH levels also significantly ($P^* < 0.001$) decreased from 3.2 ± 0.4 mU/liter at -30 min to 2.9 ± 0.4 mU/liter at 0 min ($P < 0.01$ vs. -30 min) and 2.1 ± 0.3 mU/liter at 120 min ($P < 0.02$ vs. 0 min). Mean plasma levels of LH, FSH, and hGH did not change.

ACTH (Fig. 1)

After a statistically significant fall between -30 and 0 min from 41 ± 14 to 27 ± 5 pg/ml ($P < 0.01$), mean plasma ACTH levels rose without exception ($P^* < 0.0005$). At 1.5 and 3 min, the mean values of 27 ± 4 and 26 ± 3 pg/ml, respectively, did not differ from the basal ACTH level, whereas 5 min after CRF injection, the mean plasma ACTH level was significantly higher (39 ± 6 pg/ml; $P < 0.02$). Figure 1 shows that the mean plasma ACTH level reached its maximum of 63 ± 8 pg/ml at 30 min and thereafter gradually declined to 40 ± 4 pg/ml at 240 min ($P < 0.01$ vs. 30 min). At that time, 8 of 10 subjects had ACTH levels higher than their baseline values. At 60, 120 and 180 min, the mean ACTH levels were still significantly higher than the baseline value (Fig. 1). The individual peak levels were achieved at variable intervals (from 10 to 120 min) after the administration of CRF.

Cortisol (Fig. 1)

Mean (± SEM) plasma cortisol levels decreased significantly between -30 min and 0 min from 14.4 ± 1.8 μg/100 ml (0.40 ± 0.05 μmol/liter) to 12.3 ± 1.4 μg/100 ml (0.34 ± 0.04 μmol/liter) ($P < 0.01$). After CRF administration, cortisol levels rose without exception ($P^* < 0.0005$), and at 10 min, they were significantly higher (14.1 ± 1.4 μg/100 ml; 0.39 ± 0.04 μmol/liter; $P < 0.05$) than the basal value. Figure 1 illustrates that the mean plasma cortisol level reached its maximum at 60 min (21.0 ± 0.7 μg/100 ml; 0.58 ± 0.02 μmol/liter) and thereafter gradually declined to 17.0 ± 1.1 μg/100 ml (0.47 ± 0.03 μmol/liter) at 240 min ($P < 0.05$ vs. 60 min; $P > 0.10$ vs. 0 min). Individual peak levels again were achieved at variable intervals between 20 and 180 min after CRF injection.

Aldosterone (Fig. 1)

Mean plasma aldosterone, like cortisol, decreased significantly ($P < 0.01$) between -30 and 0 min. After CRF administration, aldosterone levels rose significantly ($P^* < 0.0005$). The mean plasma aldosterone levels after CRF treatment remained unchanged through 10 min, but at 20, 30, and 60 min, aldosterone levels were significantly higher than the basal value (21 ± 3, 21 ± 2, and 23 ± 2 ng/100 ml, respectively, vs. 13 ± 2 ng/100 ml at 0 min). Figure 1

shows that the mean plasma aldosterone level reached its maximum of 23 ± 2 ng/100 ml at 60 min and thereafter gradually declined to 17 ± 2 ng/100 ml at 240 min ($P > 0.10$ vs. 0 min). Individual peak levels were achieved between 20 and 180 min after CRF injection. In 8 of the 10 subjects, the peak levels of ACTH preceded or coincided with the peak levels of cortisol and aldos-

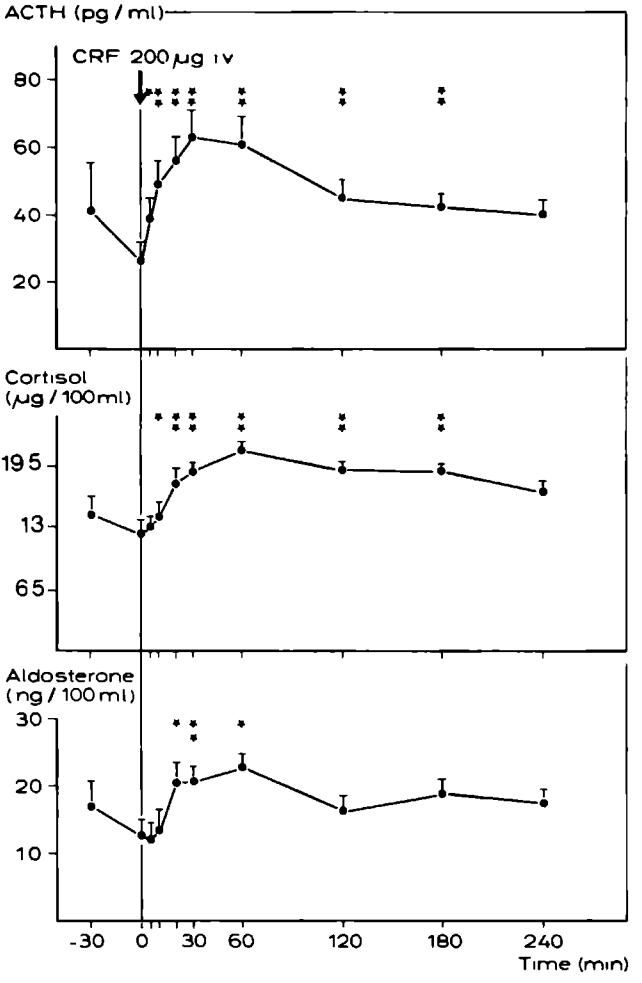

Figure 1 Mean ± SEM plasma ACTH, cortisol and aldosterone levels after an iv bolus injection of CRF (200 µg ovine CRF, Bachem) in 10 normal subjects.
The stars indicate statistical significance (*, $P < 0.05$, **, $P < 0.01$)

Table 1. ACTH, cortisol, and aldosterone levels (mean, SEM, and range) after ovine CRF administration (200 μg) in normal subjects

	Women								
	-30 min	0 min	10 min	20 min	30 min	60 min	120 min	180 min	240 min
ACTH (pg/ml)									
Mean	50	28	41	61	70	71	53	48	49
SEM	29	11	13	13	14	15	8	8	8
Range	19-166	<15-71	31-76	40-104	42-119	46-129	34-72	25-71	30-67
Cortisol (μg/100 ml)									
Mean	13.0	10.9	12.2	15.9	17.9	20.9	19.1	19.6	18.6
SEM	2.6	2.1	1.4	0.4	1.4	1.0	0.7	1.0	1.4
Range	6.6-22.4	6.6-18.1	10.1-18.1	13.3-18.1	14.4-20.4	17.9-23.1	17.2-21.8	17.2-22.9	16.1-22.4
Aldosterone (ng/100 ml)									
Mean	15	12	11	18	20	23	17	19	19
SEM	3	3	3	4	3	4	3	3	2
Range	6-26	5-23	5-23	9-34	12-33	15-39	10-23	8-25	14-24
	Men								
	-30 min	0 min	10 min	20 min	30 min	60 min	120 min	180 min	240 min
ACTH (pg/ml)									
Mean	33	26	51	51	57	51	37	38	31
SEM	5	5	12	8	12	8	4	3	3
Range	15-45	15-40	28-85	33-78	37-97	34-75	27-45	31-45	25-41
Cortisol (μg/100 ml)									
Mean	15.5	13.7	15.9	19.1	22.9	21.1	18.2	17.6	15.1
SEM	2.6	2.1	2.1	1.4	1.4	1.0	1.4	1.0	2.1
Range	8.7-23.9	7.4-19.5	9.1-19.6	14.1-22.8	15.9-21.5	19.0-25.6	13.4-22.1	14.4-21.1	12.0-17.2
Aldosterone (ng/100 ml)									
Mean	14	14	17	23	22	23	15	19	16
SEM	3	3	4	4	3	2	2	2	3
Range	2-22	3-23	6-27	14-36	13-29	16-27	8-21	10-22	9-24

terone. In the 2 other individuals, the maximal ACTH rises were the lowest of the group (16 and 24 pg/ml, respectively, vs. 56 ± 7 pg/ml in the remaining 8 subjects).

Sex differences (Table 1)

There were no significant differences between men and women in the responses of ACTH, cortisol, or aldosterone to CRF administration.

Relationships between basal cortisol and aldosterone levels and CRF-induced changes in ACTH and cortisol (Figs. 2 and 3)

When the areas under the ACTH curve were calculated from the differences between the individual ACTH values at the various time points and the individual zero values and expressed as square millimeters (1 pg/ml = 1 mm and 1 min = 1 mm), there was a highly significant and negative correlation between the areas under the individual ACTH curves and the basal cortisol levels at 0 min ($r = -0.76$, $P^{**} < 0.005$), i.e. the lower the basal plasma cortisol, the greater the rise in ACTH levels (Fig. 2). The ACTH released in response to CRF injection clearly stimulated cortisol secretion, as indicated

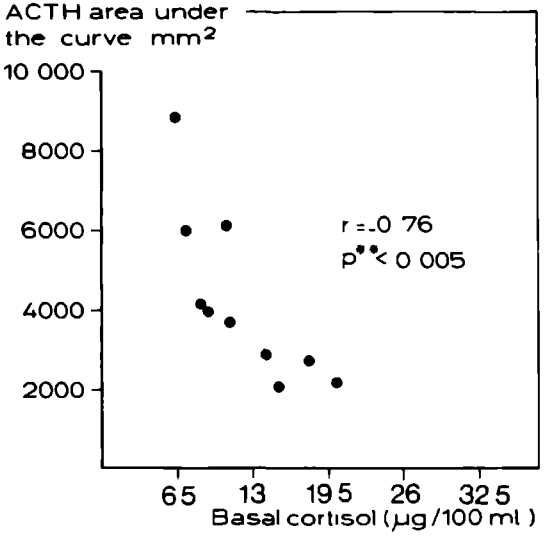

Figure 2 Intravenous bolus injection of 200 µg ovine CRF, relation between individual basal cortisol levels and the areas under the individual ACTH curves

by the highly significant correlation between the areas under the ACTH curves and those under the cortisol curves (calculated in the same way as for ACTH, 0.01 μmol/liter = 1 mm (0.36 μg/dl = 1 mm) and 1 min is 1 mm; r = 0.87; P** < 0.001). Therefore, it is not surprising that the basal cortisol levels were highly negatively correlated with the areas under the cortisol curves (r = -0.91; P** < 0.001; Fig. 3). The basal aldosterone levels also tended to be negatively related to the areas under the ACTH curves (r = -0.51; P** < 0.10). After correcting this relation for the basal cortisol levels, the resulting partial correlation coefficient between the basal aldosterone level and the area under the ACTH curve was not statistically significant (P** > 0.10). The basal levels of aldosterone were closely correlated to the basal levels of cortisol (r = 0.90; P** < 0.001). There was no significant correlation between the areas under the ACTH curves and those under the aldosterone curves (r = 0.13; P** > 0.10).

DISCUSSION

The present study is the first to demonstrate concurrent increases in plasma ACTH, cortisol, and aldosterone levels after the administration of ovine CRF to healthy subjects on a regular salt intake. In the first report on the effect of ovine CRF on ACTH and cortisol levels, Grossman et al. (4)

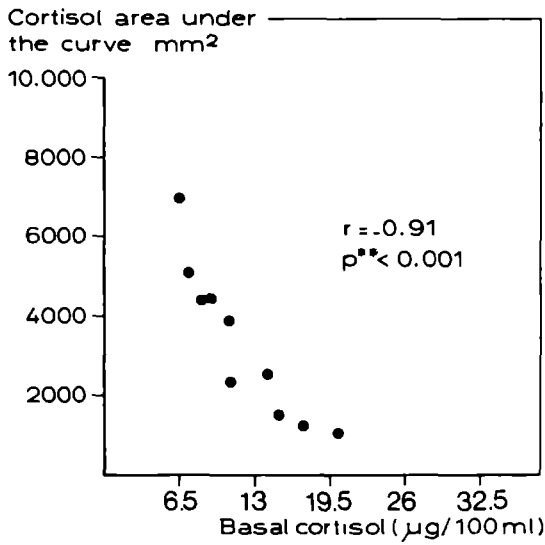

Figure 3. Intravenous bolus injection of 200 μg ovine CRF; relation between individual basal cortisol levels and the areas under the individual cortisol curves.

reported a maximal rise of ACTH levels of about 40 pg/ml and of plasma cortisol levels of about 7.2 μg/100 ml after the administration of 100 μg CRF. We found similar increases in plasma ACTH and cortisol levels using a 2-fold higher dose of CRF. Therefore, this higher dose of CRF influenced the results to only a small degree, as also can be deduced from the meticulous study by Orth et al. (6). This study further showed that the higher the basal plasma cortisol level, the smaller the CRF-induced secretion of ACTH and, subsequently, of cortisol. This intriguing observation is circumstantial evidence in favour of a role for physiological cortisol levels in the activation of the hypophyseal-adrenal axis induced by CRF and is similar to the earlier described relation between circulating T4 and the activity of the TRH-TSH axis (18). Obviously, these data do not allow any conclusion about the exact mechanism by which cortisol plays such a modulatory role. It cannot be totally excluded that the high inverse correlation between the individual basal plasma cortisol levels and the responses of the pituitary-adrenal axis to CRF is only the reflection of an inverse correlation between the individual endogenous basal CRF levels and the response of the pituitary-adrenal axis to exogenously administered CRF, i.e. in the presence of lower endogenous CRF activity exogenously administered CRF results in greater stimulation of the pituitary-adrenal axis.

In contrast to the negative modulatory effect of basal plasma cortisol levels in these normal subjects on the stimulatory effect of CRF on ACTH and subsequent cortisol secretion, in a limited number of patients with Cushing's disease, we did not find such a negative modulatory effect of basal plasma cortisol levels (8). In those patients, the stimulatory effect of CRF was greater when basal plasma cortisol levels were higher.

Another new finding of the present study was the rather brisk rise of about 20 ng/100 ml in plasma aldosterone levels 15 min after the initial ACTH increase. These data are at variance with those of Müller et al. (5), who found that 100 μg CRF had no effect on plasma aldosterone levels. Our data on aldosterone are in accordance with the known stimulatory effect of ACTH on aldosterone secretion in man (19,20).

The absence of a stimulatory effect of CRF on LH, FSH, and hGH secretion and the minor but significant fall in PRL and TSH levels strongly indicate that CRF is a specific stimulator of ACTH release.

To conclude:

1) the responses of ACTH and cortisol to CRF were inversely related to the basal plasma cortisol levels;
2) CRF elicited a rise not only in circulating ACTH and cortisol but also an increase in plasma aldosterone levels; and
3) there was no stimulatory effect of CRF on the other pituitary hormones in normal subjects.

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2.2 DIFFERENTIAL EFFECTS OF OVINE AND HUMAN CORTICOTROPHIN-RELEASING FACTOR IN HUMAN SUBJECTS

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SUMMARY

Ten healthy subjects received 200 μg of human CRF (hCRF) and 200 μg of ovine CRF (oCRF) as an intravenous bolus injection on two different occasions. After hCRF plasma ACTH levels rose significantly ($P < 0.0005$, by Friedman's nonparametric analysis of variance) from a basal value of 35 $\pm$ 3 pg/ml (mean $\pm$ SEM) to a peak value of 80 $\pm$ 7 pg/ml 30 min after hCRF administration. This ACTH response was followed by a rise in plasma cortisol levels ($P < 0.0005$, by Friedman's test) from a baseline value of 0.32 $\pm$ 0.03 μmol/l to a peak value of 0.56 $\pm$ 0.02 μmol/l 60 min after hCRF. Ovine CRF elicited similar rises in the plasma ACTH and cortisol levels. However, as derived from the faster rate of decline of ACTH and cortisol after hCRF than after oCRF, human CRF had a significantly shorter duration of action than ovine CRF in humans. Human CRF not only stimulated ACTH release by the human pituitary gland but also prolactin release. After hCRF administration prolactin levels rose significantly ($P <$ 0.005, by Friedman's test) from a basal value of 179 $\pm$ 18 mU/l to a peak value of 288 $\pm$ 34 mU/l at 10 min.

INTRODUCTION

In 1981 Vale et al. isolated and characterized a 41 amino-acid peptide from ovine hypothalami that fulfilled the criteria of a corticotrophin-releasing factor. In the last few years ample studies demonstrated that synthetic ovine CRF is a specific stimulator of ACTH release in laboratory animals (Rivier et al., 1982) and in man (Grossman et al., 1982; Orth et al., 1983; Hermus et al., 1984). In 1983 Rivier et al. showed that rat CRF, like ovine CRF a polypeptide of 41 amino-acids, differed from ovine CRF by seven amino-

acid residues. These workers also showed that rat CRF and ovine CRF have a similar potency in stimulating ACTH secretion by cultured rat pituitary cells (Rivier et al., 1983).
Very recently Shibahara et al. (1983) demonstrated the structure of human CRF by analysis of the human prepro-CRF gene. From the data of both studies one may derive that the amino-acid sequences of human CRF, as reported by Shibahara et al., and of rat CRF, as reported by Rivier et al., are identical.

The present study compares the effects of intravenous bolus injections of human and ovine CRF in a group of healthy human subjects. Our data demonstrate that both peptides elicit a similar rise of ACTH and cortisol. However, as derived from the ACTH and cortisol profiles after administration of each peptide, this study provides strong evidence that human CRF has a significantly shorter duration of action than ovine CRF in humans. Secondly, human CRF administration stimulated not only ACTH release, but also, to a minor degree, prolactin release.

SUBJECTS AND METHODS

Ten healthy subjects (eight men and two women, aged 26 ± 10 years, mean ± SD) volunteered in this study after approval of the study protocol by the hospital ethical committee. All ten subjects received 200 μg of human CRF (hCRF, Bachem, Torrance, CA) and 200 μg of ovine CRF (oCRF, Bachem) intravenously as a bolus injection on two different occasions with an interval of at least three days. Both peptides were dissolved in 2 ml acid-saline (pH 2) immediately before administration.

The CRF tests were performed at 09.00 h with the subjects fasting and in bed since 08.00 h. Blood samples for hormone assay were collected at -30, 0, 5, 10, 20, 30, 60, 120, 180, and 240 min via an indwelling i.v. forearm cannula kept open with minute amounts of a diluted (10%) solution of heparin. The cannula was inserted 1 h before CRF administration. Plasma ACTH, cortisol and human growth hormone (hGH) and serum LH, FSH, TSH and PRL were determined by specific RIA's, as described previously (Hermus et al., 1984). ACTH and cortisol levels were measured at all time intervals, both after hCRF and oCRF. Prolactin, TSH, LH, FSH and hGH levels were measured only after hCRF injection (-30, 0, 10, 30, 60 and 120 min). The areas under the individual cortisol curves were calculated by planimetry using only the parts of the curves above the baseline values. Statistical analyses were performed using Wilcoxon's paired rank test (P values denoted by P), Friedman's nonparametric analysis of variance (P = P*) and Pearson's correlation test (P = P**). The mean values ± 1 SEM are given.

RESULTS

Responses of ACTH and cortisol to human CRF administration

After a statistically significant fall between -30 and 0 min from 40 ± 3 to 35 ± 3 pg/ml ($P < 0.01$), plasma ACTH levels rose without exception ($P^* < 0.0005$) after the injection of hCRF. At 5 min plasma ACTH levels were already significantly higher (50 ± 4 pg/ml, $P < 0.01$) than the basal values.

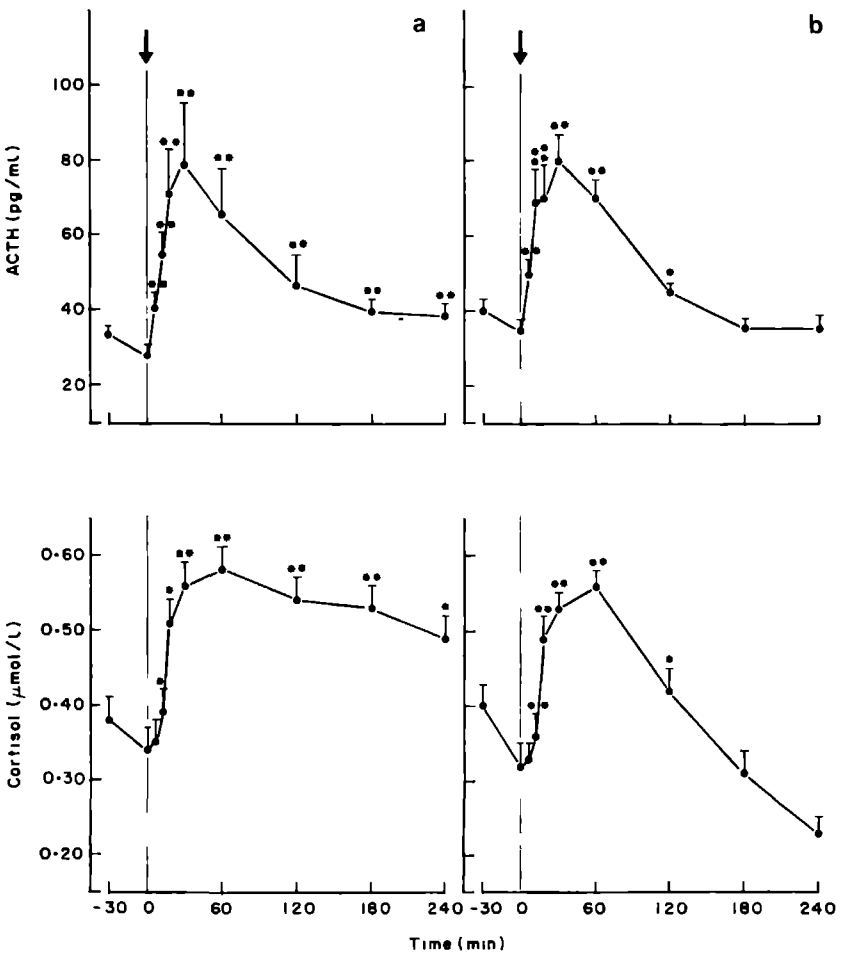

Figure 1. Mean ± SEM plasma ACTH and cortisol levels after i.v. bolus injections of a. 200 μg ovine CRF and of b. 200 μg human CRF in 10 healthy subjects. Arrows mark injection. Statistical significance, $^*P < 0.05$, $^{**}P < 0.01$.

Figure 1 shows that the plasma ACTH level reached its maximum of 80 ± 7 pg/ml at 30 min and thereafter rapidly declined to 36 ± 2 pg/ml at 180 min ($P < 0.01$ vs 30 min) and 36 ± 3 pg/ml at 240 min ($P < 0.01$ vs 30 min). The ACTH concentrations at 180 and 240 min did not differ significantly from the basal ACTH concentrations. The individual peak levels were achieved at variable intervals (from 10 to 60 min) after the administration of hCRF.

Plasma cortisol levels decreased significantly between -30 min and 0 min from 0.40 ± 0.03 μmol/litre to 0.32 ± 0.03 μmol/litre ($P < 0.01$) before hCRF injection. Thereafter, cortisol values rose without exception ($P^* <$

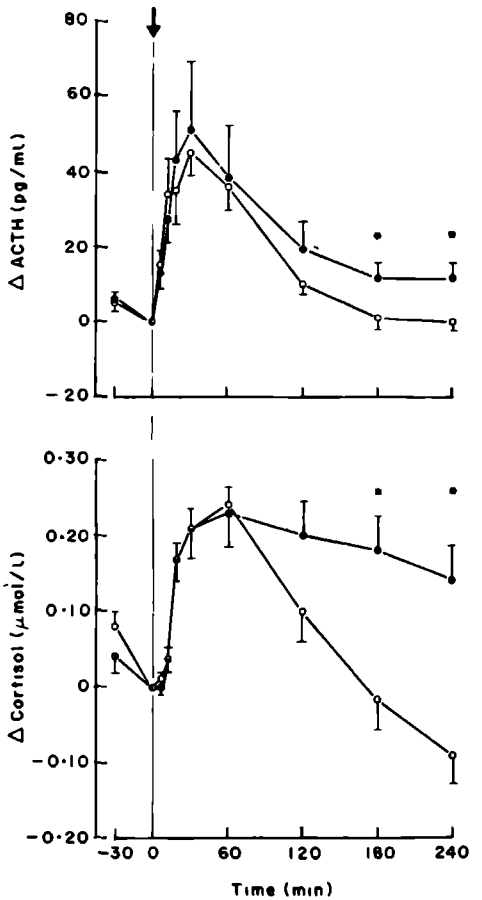

Figure 2. Changes (Δ) in the concentrations of plasma ACTH and cortisol after the i.v. administration of 200 μg ovine CRF (●) and 200 μg human CRF (○) to 10 healthy subjects. The mean values ± SEM are given. Significant differences between the changes after oCRF and hCRF, $^*P < 0.05$.

0.0005) and at 10 min they were significantly higher (0.36 ± 0.03 μmol/l, $P < 0.01$) than the basal values. Figure 1 illustrates that plasma cortisol reached its maximum at 60 min (0.56 ± 0.02 μmol/l) and thereafter very rapidly declined to 0.23 ± 0.02 μmol/l at 240 min ($P < 0.01$ vs 60 min, $P < 0.05$ vs 0 min). At that time, all subjects except two had cortisol concentrations lower than their baseline values. Individual peak levels were achieved again at variable intervals between 20 and 60 min after hCRF injection.
In seven of the 10 subjects the cortisol peak was achieved at a later time than the ACTH peak and in three subjects at the same time.

Responses of ACTH and cortisol to ovine CRF administration

After oCRF injection ACTH levels rose without exception ($P^* < 0.0005$) from a basal level of 28 ± 3 pg/ml to a peak level of 79 ± 16 pg/ml at 30 min. Figure 1 shows that the plasma ACTH values at 180 and 240 min after oCRF administration, in contrast to those after hCRF, were still significantly higher than the baseline values. This ACTH response was followed by a rise in plasma cortisol levels ($P^* < 0.0005$) except in one subject, who had the highest basal cortisol level of the group. Cortisol rose from a basal level of 0.34 ± 0.03 μmol/l to a peak of 0.58 ± 0.03 μmol/l at 60 min.

Figure 1 clearly shows that the plasma cortisol concentrations at 180 and 240 min after oCRF administration in contrast to those after hCRF were still significantly higher than the baseline values.

Comparison between the ACTH and cortisol responses after hCRF and oCRF

Figure 2 shows that hCRF and oCRF elicited similar rises in the plasma ACTH and cortisol levels. Maximum ACTH levels were reached at 30 min after both oCRF and hCRF. Thereafter both ACTH curves ran a significantly dissociated course.
At 180 and 240 min the ACTH levels after hCRF were significantly lower than the corresponding levels after oCRF (both $P < 0.05$). Maximum cortisol levels were reached at 60 min, after both oCRF and hCRF. Thereafter, like the ACTH curves, both cortisol curves ran a significantly dissociated course. At 180 and 240 min the cortisol levels after hCRF were significantly lower than the corresponding levels after oCRF (both $P < 0.05$). At 240 min the plasma cortisol levels after oCRF injection were still higher ($P < 0.05$) than the basal values, whereas after hCRF administration these levels were significantly lower ($P < 0.05$) than the basal levels.

There was a significantly negative correlation between the basal cortisol concentrations and the areas under the individual cortisol curves, both after hCRF ($r = -0.80$; $P^{**} < 0.01$) and oCRF ($r = -0.80$; $P^{**} < 0.01$).

Responses of PRL, TSH, LH, FSH and hGH to human CRF administration

After a statistically insignificant fall between -30 and 0 min from 213 ± 28 mU/l to 179 ± 18 mU/l prolactin levels rose without exception ($P^* < 0.005$). The prolactin level reached its maximum of 288 ± 34 mU/l at 10 min ($P < 0.01$ vs 0 min) and thereafter gradually declined to 113 ± 8 mU/l at 120 min ($P < 0.01$ vs 10 min, $P < 0.02$ vs 0 min). At that time, seven of nine subjects had prolactin levels lower than their baseline values. The individual peak levels for prolactin were achieved at variable intervals (from 10 to 60 min) after the administration of hCRF. The concentrations of hGH, TSH, LH and FSH remained unchanged after hCRF injection.

Side effects

After administration of both hCRF and oCRF flushing and mild dyspnoea (subjective sensation of shortness of breath, accompanied by the objective finding of deep and more rapid respirations, lasting 30 sec or less) occurred in most subjects. Mean diastolic blood pressure was significantly lowered after both oCRF and hCRF (Hermus et al., 1983b). Major complications did not occur in these healthy subjects (Hermus et al., 1983a).

DISCUSSION

Human CRF and ovine CRF, both peptides of 41 amino-acids, differ in seven amino-acid residues, six of seven replacements occurring in the carboxy-terminal half of the molecule. Rivier et al. (1983) have demonstrated that rat CRF, which has an amino-acid sequence identical to human CRF, is equipotent to ovine CRF in stimulating ACTH release from rat pituitary cells in monolayer culture. However, so far no data have been published systematically comparing the effects of human and ovine CRF in humans in vivo.

The present study demonstrates that human CRF, like ovine CRF, stimulates ACTH and thereby cortisol release in healthy human subjects and that both releasing factors elicit similar increases of plasma ACTH and cortisol after an intravenous bolus injection of 200 μg. Furthermore, we show that the cortisol response after human CRF administration is negatively correlated with the basal cortisol concentration, as has already been noted for ovine CRF by Hermus et al. (1984) and Lytras et al. (1984). However, this study provides strong evidence that human CRF has a much shorter duration of action than ovine CRF in man. After ovine CRF administration plasma cortisol levels remained significantly elevated until 240 min. In contrast, after human CRF, the cortisol levels had already returned to baseline values 180 min after injection of the peptide, whereas at

240 min the plasma cortisol levels were even significantly lower than the basal concentrations. Comparing the ACTH profiles after injection of each peptide, a similar statistically significant divergence can be observed, i.e. at 180 and 240 min the ACTH levels after hCRF were significantly lower than the corresponding levels after oCRF.

It has clearly been demonstrated in laboratory animals and in man that ovine CRF, when injected as an intravenous bolus, has a remarkably long plasma half-life compared with those of TRH (Morley et al., 1979) and LHRH (Pimstone et al., 1977). There is no doubt that this long half-life is responsible for the prolonged biological effects of ovine CRF injected intravenously. In man Nicholson et al. (1983) and Tsukada et al. (1984) calculated that IR-ovine CRF disappeared biexponentially after a single intravenous bolus injection following a decay curve characterized by $t^1/_2$ values of 6 min for the fast phase and of 50 min for the slow phase. Our data do not allow an estimation of the disappearance half-time of human CRF. However, the much shorter duration of hypophyseal-adrenal axis stimulation after human CRF in comparison to after ovine CRF injection strongly suggests a shorter half-life for human CRF. Such shorter duration of action would also be more in line with those of other releasing hormones such as TRH and LHRH.

Another interesting observation in this study is that human CRF stimulates prolactin secretion in humans although to a minor degree. This is in contrast to ovine CRF administration (Grossman et al., 1982; Hermus et al., 1984). In an earlier study from our laboratory (Hermus et al., 1984) prolactin levels significantly decreased after ovine CRF injection. In sheep (Donald et al., 1983) ovine CRF is unable to stimulate prolactin secretion. However, Schulte et al. showed that ovine CRF caused dose-dependent prolactin and even growth hormone responses in cynomolgus (Schulte et al., 1982) and rhesus monkeys (Schulte et al., 1983). In the rhesus monkey it was also demonstrated that pretreatment and simultaneous treatment with naloxone inhibited both growth hormone and prolactin responses without affecting the activation of the pituitary-adrenal axis by ovine CRF (Schulte et al., 1983). Obviously our data do not allow any conclusion about the mechanism of this slight prolactin release by human CRF.

Human and ovine CRF differ in 17% of the amino-acid residues. This marked nonalignment between releasing factors of different mammalian species has not been seen for the smaller hypothalamic releasing factors. Our study indicates that these structural differences have an important effect on biological activity, as was predicted on theoretical grounds by Rivier et al. (1983).

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2.3 HYPOTENSIVE EFFECTS OF OVINE AND HUMAN CORTICOTROPHIN-RELEASING FACTOR IN MAN

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SUMMARY

The hemodynamic effects of i.v. bolus injections of 100 and 200 μg ovine or human CRF in man were compared. After 100 μg ovine CRF as well as after 100 μg human CRF no significant change occurred in blood pressure. However, in all individuals pulse rate increased. The mean maximum increase in pulse rate after hCRF was almost twice that after oCRF (21 ± 5 vs 12 ± 4 beats/min; $P^* < 0{,}05$). After 200 μg oCRF diastolic blood pressure declined gradually in all subjects from 77 ± 3 mmHg to a nadir of 67 ± 3 mmHg at 22 min ($P < 0{,}002$). After 200 μg hCRF diastolic blood pressure decreased more precipitously from 78 ± 3 mmHg to a nadir of 61 ± 3 mmHg at 6 min ($P < 0{,}002$). The fall in diastolic blood pressure after hCRF was significantly greater than after oCRF ($P^* < 0{,}05$). After 200 μg oCRF a slight increase in pulse rate was found, lasting for 6 minutes. However, after 200 μg hCRF marked (reflex)tachycardia occurred lasting for 30 minutes. Only after the highest dose of hCRF a slight increase in systolic blood pressure occurred. Hemodynamic effects after both doses of human CRF were accompanied by significant increases in plasma norepinephrine levels, which were significantly more pronounced after the higher dose.

INTRODUCTION

Several studies have claimed that the administration of corticotrophin-releasing factor can unravel the cause of hyper- and hypocortisolemic conditions in man (Chrousos et al 1984; Müller et al 1983; Tsukada et al 1984; Hermus et al 1985). So far, in most studies ovine CRF, which became available first, has been used. Human CRF - like ovine CRF a polypeptide of 41 amino-acids - differing from the latter by 7 amino-acid residues, would be preferable on theoretical grounds, especially because of its shorter duration

Submitted for publication

of action (Hermus et al 1984), caused by its shorter plasma half-life (Schürmeyer et al 1984).

Ovine CRF, like the non-mammalian CRF-like peptides sauvagine and urotensin I, causes hypotension and tachycardia when injected intravenously in rats (Fisher et al 1983), dogs (MacCannell et al 1982; Brown et al 1982; Brown and Fisher 1983), rhesus monkeys (Kalin et al 1982) and humans (Orth et al 1983; Hermus et al 1983), but not in sheep (Kalin et al 1983a; Scoggins et al 1984).

A systematic study comparing the hemodynamic effects of ovine and human CRF in man is not available. The present study demonstrates that both human and ovine CRF have a significant hypotensive effect in man, which is most pronounced for the human peptide. In addition we show that human CRF causes a dose-dependent rise of plasma norepinephrine and not of epinephrine levels, most probably reflecting activation of the sympathetic nervous system.

SUBJECTS AND METHODS

Thirty-one healthy subjects (20 men and 11 women) volunteered for this study after approval of the study protocol by the hospital ethical committee. Informed consent was obtained from all participants. Twenty-five subjects were studied once and six twice. The volunteers were divided in four groups. Group I ($n = 10$, age 42 ± 12 years, mean $\pm$ SD) received 100 μg ovine CRF, group II ($n = 8$, age 23 ± 4 years) 100 μg human CRF, group III ($n = 10$, age 27 ± 9 years) 200 μg ovine CRF and group IV ($n = 9$, age 24 ± 7 years) 200 μg human CRF.

Ovine CRF (oCRF) and human CRF (hCRF) were obtained from Bachem Inc. (Torrance, CA). CRF was dissolved in a 2% aqueous solution of lactose and sterilized by passage through a 0,2 μm cellulose acetate filter (Schleicher und Schuell, FPO 30/3) and was found to be pyrogen free ('Pyrogent test', Mallinckrodt). CRF was stored at -18°C in lyophilized form in sterile vials under vacuum. Immediately before administration lyophilized material was dissolved in 1-2 ml acid-saline (pH 2).

CRF was injected at 09.00 h with the subjects supine since 08.00 h after an overnight fast. Blood samples for hormone assays were collected at -30, 0, 5, 10, 20, 30, 60, 120, 180 and 240 min via an indwelling i.v. forearm cannula kept open with a diluted solution of heparin. The cannula was inserted 1 h before the administration of CRF as an i.v. bolus injection of 30 seconds. Blood pressure and pulse rate were measured every two minutes from 10 minutes before to 30 minutes after CRF administration and afterwards at 15 minute intervals for 240 minutes after CRF injection (Roche 'Arteriosonde' 1225, monitor 103). The mean values calculated from 5 consecutive measurements before CRF administration were considered as the basal

values. In 7 subjects of group II (100 μg hCRF) and in 6 subjects of group IV (200 μg hCRF) plasma norepinephrine and epinephrine levels were determined at -30, 0, 5, 10, 20, 30, 60 and 120 min, using a specific radioenzymatic assay as described previously (Hoffmann et al 1982) with some modifications. Mean responses at each point of time after CRF administration were compared with the mean basal value by Student's t-test for paired observations (P values denoted by P). In order to reduce the overall probability of a type I error to $P < 0{,}03$ a level of significance of $P < 0{,}002$ was employed (Bonferroni's correction). In addition, statistical analyses were performed using Wilcoxon's two sample test ($P = P^*$), and Friedman's nonparametric analysis of variance ($P = P^{**}$) and by testing the means of Spearman's rank correlation coefficients ($P = P^{***}$). Mean values $\pm$ 1 SEM are given.

RESULTS

Basal blood pressure and pulse rate did not differ between the four groups. Figures 1 and 2 illustrate the changes in mean blood pressure and pulse rate during the first 30 minutes following CRF injection. After that time the mean blood pressure and pulse rate did not differ from the baseline.

Figure 1 shows that no significant changes in systolic or diástolic blood pressure occurred after either 100 μg ovine or human CRF. In all individuals pulse rate increased after administration of both CRF's, and the mean peak increases in pulse rate were twice as high after hCRF as after oCRF (21 ± 5 vs. 12 ± 4 beats/min; $P^* < 0{,}05$). Individual peak levels were achieved at a median time of 6 min (range 2-28 min) after 100 μg oCRF and at 3 min (range 2-18 min) after 100 μg hCRF. In two subjects, one in the oCRF and one in the hCRF group pulse rate increased dramatically by 50 and 59 beats/min respectively.

After 200 μg oCRF (Figure 2), diastolic blood pressure dropped in all subjects from 77 ± 3 mmHg to a nadir of 67 ± 3 mmHg at 22 min ($P < 0{,}002$). Individual nadirs were achieved at a median time of 14 min (range 2-24 min) and individual decreases in blood pressure varied widely between 3 and 25 mmHg (mean 14 ± 2 mmHg). After 200 μg hCRF diastolic blood pressure declined more sharply from 78 ± 3 mmHg to a nadir of 61 ± 3 mmHg at 6 min ($P < 0{,}002$). Individual nadirs were achieved at a median time of 4 min (range 2-26 min) and individual decreases in blood pressure varied between 12 and 34 mmHg (mean 23 ± 2 mmHg). The mean decrease of diastolic blood pressure in the first 10 min after 200 μg hCRF was significantly larger than after 200 μg oCRF ($P^* < 0{,}05$). Figure 2 also illustrates that systolic blood pressure rose after 200 μg hCRF, but not after 200 μg oCRF. Pulse rate increased in all subjects after 200 μg oCRF. Individual peak levels were achieved at a median time of 7 min (range 2-28 min) and the individual peak

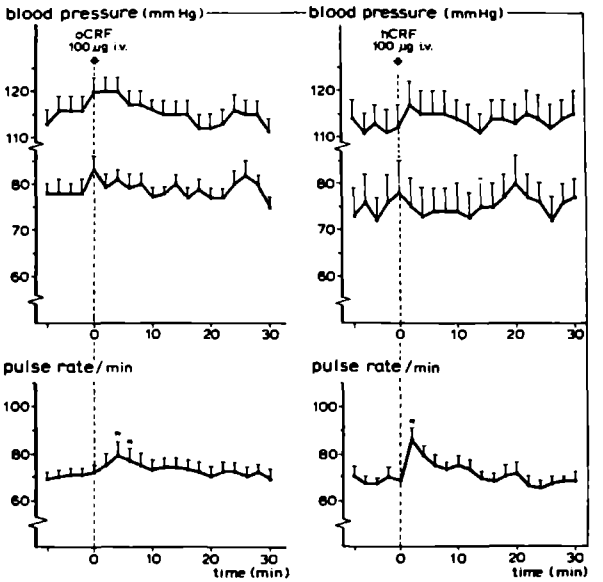

Figure 1. Response of blood pressure and pulse rate (mean ± SEM) after a bolus injection of 100 µg oCRF (left panel; n = 10) and 100 µg hCRF (right panel; n = 8). Asterisks, statistical significance (P < 0,002) vs. basal value.

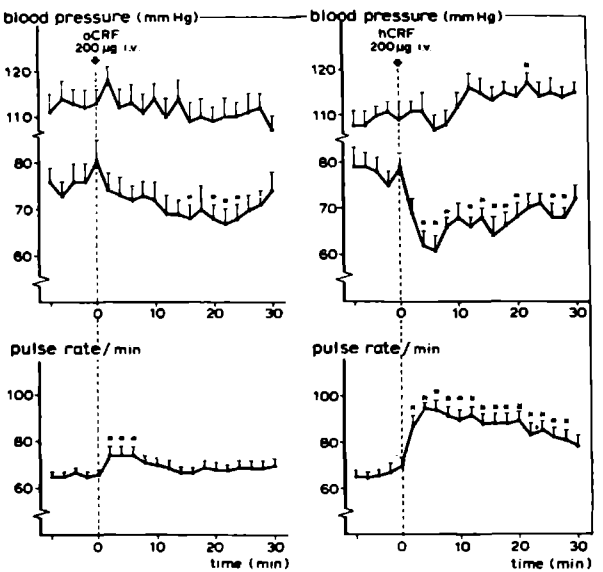

Figure 2. Response of blood pressure and pulse rate (mean ± SEM) after a bolus injection of 200 µg oCRF (left panel; n = 10) and 200 µg hCRF (right panel; n = 9). Asterisks, statistical significance (P < 0,002) vs. basal value.

increases varied widely between 2 and 27 beats/min (mean 16 ± 3 beats/min). After 200 μg hCRF marked tachycardia occurred in all individuals. Individual peak levels were achieved at a median time of 10 min (range 4-24 min) and the individual peak increases varied between 15 and 52 beats/min (mean 35 ± 4 beats/min). The mean increase of pulse rate in the first 10 min after 200 μg hCRF was significantly higher than after 200 μg oCRF ($P^* < 0,01$). The individual changes in diastolic blood pressure and in pulse rate in the first 4 minutes after CRF were significantly and inversely correlated, both after 200 μg oCRF ($r = -0,49$; $P^{***} < 0,05$) and 200 μg hCRF ($r = -0,69$; $P^{***} < 0,01$).

Figure 3 gives the results of the plasma catecholamine levels after 100 and 200 μg hCRF administration. After 100 μg hCRF plasma norepinephrine rose significantly ($P^{**} < 0,025$) from a basal level (= the mean of the -30 and 0 value in each subject) of 1,05 ± 0,14 nM/l to a peak level of 1,24 ± 0,17 nM/l at 10 min, whereas after 200 μg hCRF plasma norepinephrine increased significantly ($P^{**} < 0,025$) from a basal level of 1,19 ± 0,20 nM/l to a peak level of 2,11 ± 0,49 nM/l at 10 min. Maximum norepinephrine rises following 200 μg hCRF were significantly ($P^* < 0,05$) higher than after 100 μg hCRF. Plasma epinephrine levels did not change after either dose of hCRF.

DISCUSSION

The present study demonstrates that in healthy subjects bolus injections of 200 μg of both ovine and human CRF cause a significant decrease in diastolic blood pressure, which was not observed after the 100 μg doses. This study also shows, for the first time, that human CRF causes greater hypotension than ovine CRF in man.

After the administration of 100 μg ovine or human CRF in all individuals pulse rate increased without significant changes in blood pressure. Maximum increase in pulse rate after hCRF was almost twice that of oCRF. After the administration of the higher dose of ovine CRF diastolic blood pressure declined gradually in all individuals, whereas after the same dose of human CRF diastolic blood pressure dropped more abruptly and to a significantly lower level. Again, hCRF accelerated the pulse rate significantly more than the ovine peptide. The significant and inverse correlation between the decline in diastolic blood pressure and the increase in pulse rate during the first four minutes after the injection of 200 μg CRF, strongly suggests activation of the sympathetic nervous system.

In laboratory animals ovine CRF causes hypotension and tachycardia when injected intravenously in rodents (Fisher et al 1983), dogs (MacCannell et al 1982; Brown et al 1982; Brown and Fisher 1983) and rhesus monkeys (Kalin et al 1982), but remarkably, not in sheep (Kalin et al 1983a, Scoggins

et al 1984). In contrast, intracerebroventricular administration of ovine CRF causes increases in blood pressure and heart rate in rodents (Fisher et al 1982; Fisher et al 1983),.dogs (Brown and Fisher 1983), rhesus monkeys (Kalin et al 1983b) and also in sheep (Scoggins et al 1984).

Data published about hemodynamic effects of CRF in man is scarce. Orth et al (1983) measured pulse rate and blood pressure at frequent intervals after *ovine* CRF injection and found an increase in pulse rate after 3 μg/kg oCRF comparable with our data, whereas marked tachycardia (pulse rate increased with 35 beats/min) lasting for more than 30 min occurred after 30 μg/kg oCRF. In the limited number of subjects they tested, a significant decline in

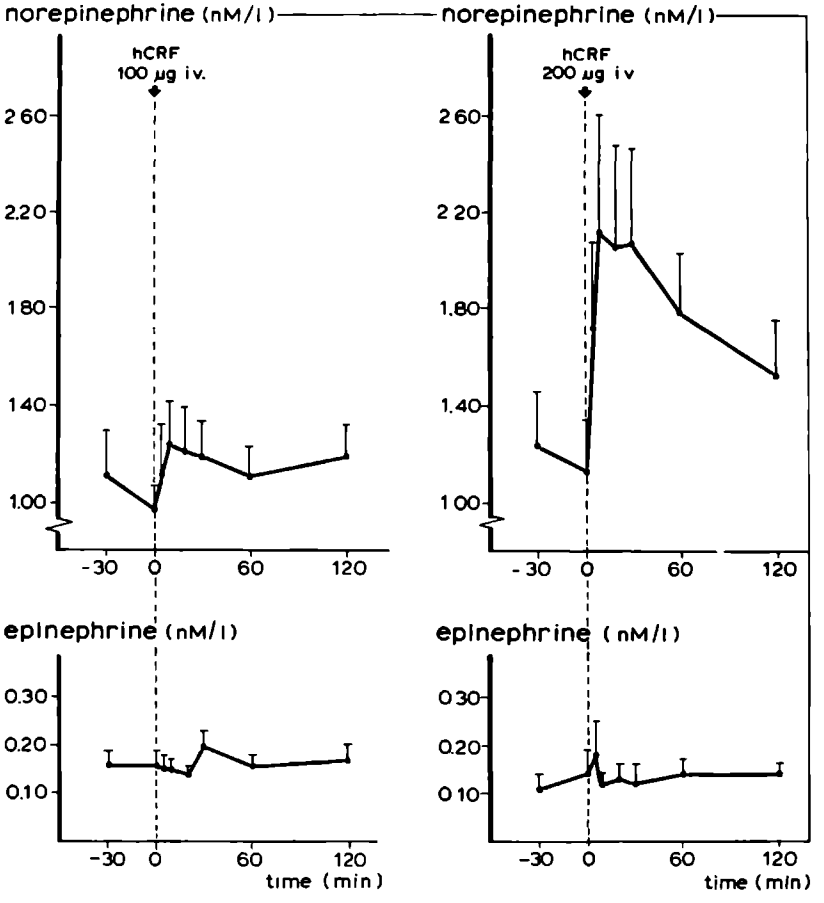

Figure 3. Plasma epinephrine and norepinephrine levels (mean ± SEM) after a bolus injection of 100 μg hCRF (left panel) and 200 μg hCRF (right panel).

diastolic blood pressure could only be demonstrated after 30 μg/kg oCRF. However, individual subjects showed decreases in mean blood pressure of 15-29 mmHg after 10 or 30 μg/kg oCRF, despite recumbency and replacement of lost volume. In contrast to the present study Schürmeyer et al (1984), using CRF from the same source as ours, found no significant effect on blood pressure, after even 5 μg/kg *human* CRF, although pulse rate increased by 26%. However, these authors measured blood pressure and pulse rate only at 15-30 min intervals with baseline values based on only two measurements.

The present study also demonstrates significant rises in plasma norepinephrine levels immediately after human CRF administration, which were highest after the higher dose, whereas plasma epinephrine did not change. The hemodynamic profile of CRF in man together with the rise of norepinephrine levels fits well into the concept that the hypotensive action of CRF in mammals is caused by vasodilatation of resistance vessels (MacCannell et al 1982).

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2 4 ESCAPE FROM DEXAMETHASONE INDUCED ACTH AND CORTISOL SUPPRESSION BY CORTICOTROPIN-RELEASING HORMONE (CRH) MODULATORY EFFECT OF BASAL DEXAMETHASONE LEVELS[1]

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SUMMARY

The response of ACTH and cortisol to CRH after pretreatment with various doses of dexamethasone was investigated in five healthy subjects. The five subjects participated in six experiments. In each experiment 200 μg ovine CRH was administered as an i.v. bolus injection at 09.00 h after pretreatment with respectively: A) 1 mg dexamethasone orally at 23.00 h on the evening before CRH injection, B) idem, 2 mg dexamethasone, C) idem, 4 mg dexamethasone, D) idem, 2 mg dexamethasone, followed by 2 mg dexamethasone orally 1 hour before CRH , E) no dexamethasone and F) 1 mg dexamethasone orally 1 hour before CRH injection. In spite of overnight suppression with dexamethasone CRH elicited cortisol rises in all individuals (experiments A to C). Dexamethasone pretreatment in experiment D abolished the CRH induced stimulation of the pituitary-adrenal axis. There was a significant and negative correlation between the basal dexamethasone levels (i.e. the dexamethasone levels immediately before CRH administration) in the experiments A to D and the areas under the individual ACTH ($r = -0.62$; $P < 0.01$ by Spearman's rank correlation test) and cortisol ($r = -0.81$; $P < 0.001$ by Spearman's test) curves, i.e. the lower the basal dexamethasone levels, the greater the rise in ACTH and cortisol levels after CRH administration. Pretreatment with a single dose of 1 mg dexamethasone 1 hour before CRH injection (experiment F) led to a significant inhibition of the CRH induced ACTH and cortisol response, despite unsuppressed pre-CRH ACTH and cortisol levels.

[1] Presented in part at the Second Meeting of the European Neuroendocrine Association (Abstract 133), Milan, October 15-17, 1985

Submitted for publication

We conclude that CRH is able to overrule the inhibition of the pituitary-adrenal axis by overnight suppression with pharmacological doses of dexamethasone. The strongly negative correlation between the basal dexamethasone levels and the CRH induced pituitary-adrenal responses argues for the primacy of circulating glucocorticoid levels in the modulation of the response of ACTH and cortisol to CRH.

INTRODUCTION

In previous studies (1,2) we have shown that the response of the pituitary-adrenal axis to exogenous CRH in healthy man highly depends on the individual basal cortisol level, i.e. the higher the basal plasma cortisol level, the lower the response of ACTH and cortisol after CRH administration. At present it is not known by which mechanism the basal cortisol level modulates the response to CRH. It is attractive to assume that the basal cortisol levels themselves determine the ACTH response to CRH by their negative feedback to the corticotrophs. However, the possibility remains that the observed correlation between the individual basal cortisol levels and the ACTH and cortisol responses to exogenous CRH is in fact a spurious one, primarily dictated by an underlying correlation between the individual endogenous CRH levels and the response of the pituitary to exogenous CRH.

In the present study we demonstrate a highly significant and negative correlation between the plasma ACTH and cortisol responses to CRH in healthy subjects after overnight suppression with pharmacological doses of dexamethasone, and the plasma level of the synthetic glucocorticoid immediately before injection of CRH. It is known from studies in rodents (3-7) that pharmacological doses of dexamethasone suppress endogenous CRH activity. Therefore, the demonstration of a negative correlation between the level of this synthetic glucocorticoid and the response of the pituitary-adrenal axis to CRH provides an argument that the level of circulating glucocorticoids per se determines the response to CRH in man.

SUBJECTS AND METHODS

Five healthy subjects (four men and one woman, aged 23 ± 2 yr, mean $\pm$ SD) participated in this study. Informed consent was obtained from all volunteers after approval of the study protocol by the hospital ethical committee. The five subjects participated in six experiments. In each experiment 200 μg ovine CRH (Bachem, Torrance, CA) was administered as an intravenous bolus injection at 09.00 h after pretreatment with, respectively:

A) 1 mg dexamethasone (Oradexon, Organon, Oss) orally at 23.00 h on the evening before CRH injection.
B) idem, 2 mg dexamethasone
C) idem, 4 mg dexamethasone
D) idem, 2 mg dexamethasone, followed by 2 mg dexamethasone orally 1 hour before CRH injection
E) no dexamethasone
F) 1 mg dexamethasone orally 1 hour before CRH injection

Moreover, in a seventh experiment three of the five subjects received 2 ml saline, instead of CRH, after pretreatment with 1 mg dexamethasone at 23.00 h on the evening before the experiment. All experiments were separated from each other by at least 4 days. Ovine CRH was dissolved in 2 ml acid-saline (pH 2) immediately before administration. All tests were performed after an overnight fast with the subjects supine since 08.00 h. Blood samples for plasma ACTH and cortisol assay were collected at -30, 0, 5, 10, 20, 30, 60, 120, 180, and 240 min through an indwelling i.v. cannula kept open with small amounts of a diluted (10%) solution of heparin. The cannula was inserted 1 hour before CRH (or saline) administration.

Plasma ACTH and cortisol were measured by specific RIA's as described previously (8). Plasma dexamethasone levels were measured by radioimmunoassay after extraction with dichloromethane and paperchromatographic purification in a Bush C type solvent system (methanol: toluol: ethylacetate: water = 250:450:50:250; Rf DXM = 0,35). The antiserum (kindly donated by Prof.P.Vecsei, University of Heidelberg, FRG) was raised in rabbits against dexamethasone-3 carboxy-methyloxime coupled to bovine serum albumine. ^{3}H-dexamethasone was used as the tracer (NEN, NET 467). At the extremely low plasma levels of cortisol during suppression with dexamethasone no interference of cortisol with the antibody to dexamethasone (cross-reactivity 0,05%) can be expected. The mean intra-assay coefficient of variation of a plasma pool was 6,5% (mean level 252 ng/100 ml, n = 10). The interassay coefficient of variation was 8,3% (mean level 258 ng/100 ml, n = 8). To avoid this interassay variation all samples were measured in the same assay.

The areas under the individual ACTH and cortisol curves were calculated by planimetry using only the parts of the curves above the baseline values. (ACTH: 1 pg/ml = 1 mm; cortisol: 0.01 μmol/l = 0.362 μg/100 ml = 1 mm; 1 min = 1 mm).

Statistical analysis was performed using Student's t-test for paired observations (P values denoted by P), Friedman's nonparametric analysis of variance (P = P*) and Spearman's rank correlation test (P = P**). Unless otherwise stated, the mean values ± 1 SEM are given.

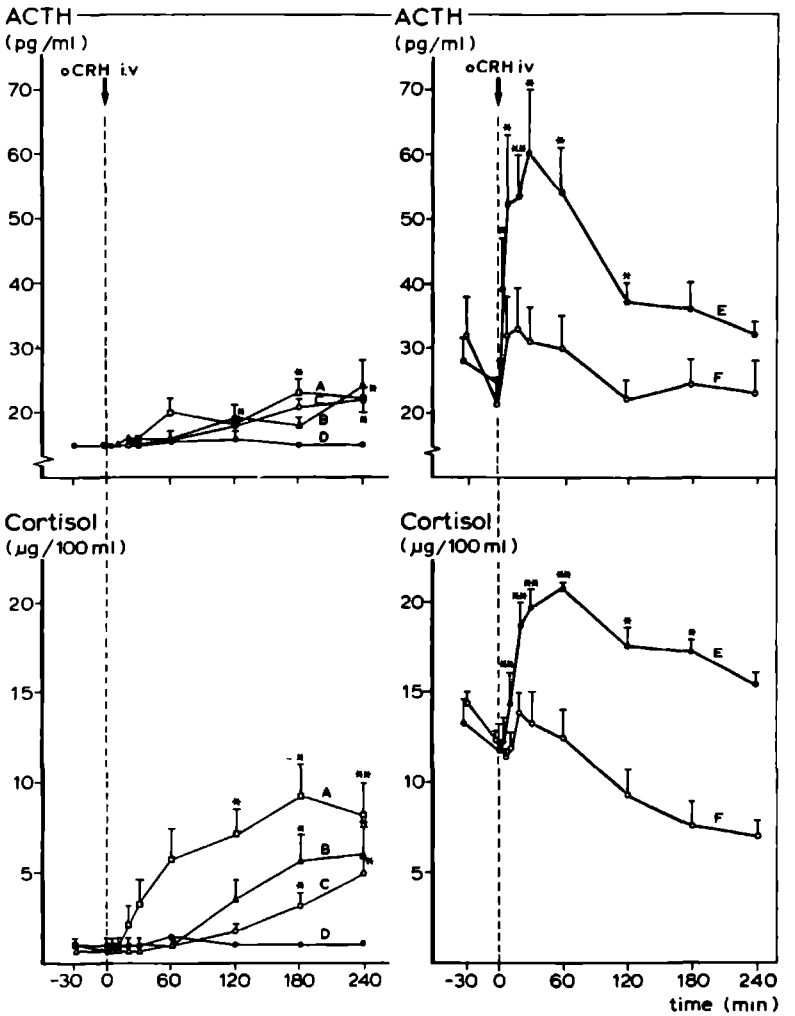

Figure 1. Mean ± SEM plasma ACTH and cortisol responses to an i.v. bolus injection of 200 µg ovine CRH at 09.00 h in 5 normal subjects after pretreatment with, respectively:

A) 1 mg dexamethasone orally at 23.00 h on the evening before CRH injection
B) idem, 2 mg dexamethasone
C) idem, 4 mg dexamethasone
D) idem, 2 mg dexamethasone, followed by 2 mg dexamethasone orally 1 hour before CRH injection
E) no dexamethasone
F) 1 mg dexamethasone orally 1 hour before CRH injection.

The asterisks indicate statistical significance (* $P < 0.05$; ** $P < 0.01$).

RESULTS

1. Suppression of the ACTH and cortisol responses to CRH after pretreatment with dexamethasone

1.1 *Responses of ACTH and cortisol to CRH after overnight suppression with dexamethasone (experiments A to D)*

Figure 1 (left panel) illustrates the mean ACTH and cortisol responses to a bolus injection of 200 μg ovine CRH after overnight suppression with increasing doses of dexamethasone. In all experiments overnight suppression with dexamethasone resulted in ACTH values below the detection limit of the assay (15 pg/ml), whereas all basal cortisol values were 1,4 μg/100 ml or less. The figure clearly shows that in spite of overnight suppression with 1 mg dexamethasone, CRH induced a marked cortisol rise ($P^* < 0.001$) in all individuals with a mean peak value of 9,4 ± 1,8 μg/100 ml at 180 min. After overnight suppression with 2 mg or even 4 mg dexamethasone, CRH was still able to raise cortisol levels in all individuals (experiment B, $P^* < 0.01$; experiment C, $P^* < 0.025$).

It also appears that overnight suppression with dexamethasone changes the time-course of the cortisol response, i.e. the CRH induced increase of the cortisol level after dexamethasone pretreatment is slower in comparison with the rather brisk rise of plasma cortisol without dexamethasone pretreatment (experiment E, vide infra). Without dexamethasone pretreatment a significant increase of plasma cortisol was reached at 10 min, whereas after overnight treatment with dexamethasone, the increases of plasma cortisol reached statistical significance only after 120 min (experiment A) and 180 min (experiments B and C).

The ACTH values, measured in the experiments A to C are lower than or near to the detection limit of the assay. In the experiments A and C a significant ACTH rise preceded the rise in plasma cortisol. The figure also shows that 2 mg dexamethasone given orally one hour before CRH injection in addition to 2 mg dexamethasone at 23.00 h on the previous evening (experiment D) abolished the CRH induced stimulation of the pituitary-adrenal axis. Saline injection instead of CRH after overnight suppression with 1 mg dexamethasone did not produce an increase in the suppressed ACTH (< 15 pg/ml) and cortisol (< 0,7 μg/1υ0 ml) levels in the three individuals tested until 240 min after the injection (data not shown). This proves that the ACTH and cortisol responses observed in the experiments A to C were not due to cessation of the suppressive effect of dexamethasone and excluded aspecific stress as the cause of pituitary-adrenal activation in these experiments.

1.2 *Responses of ACTH and cortisol to CRH after pretreatment with dexamethasone in the presence of unsuppressed ACTH and cortisol levels (experiment E and F)*

Figure 1 (right panel) illustrates the ACTH and cortisol responses to a bolus injection of 200 μg ovine CRH with and without pretreatment with 1 mg dexamethasone given orally one hour before CRH injection. Basal ACTH and cortisol levels did not differ significantly in either experiment.
After dexamethasone pretreatment (experiment F) the initial rise of plasma ACTH and cortisol was not statistically significant. The maximal ACTH (13 $\pm$ 5 vs 44 $\pm$ 5 pg/ml; $P < 0.05$) and cortisol (2.5 $\pm$ 1.1 vs 9.8 $\pm$ 1.1 μg/100 ml; $P < 0.005$) increments after CRH were significantly lower after 1 mg dexamethasone one hour before CRH. The integrated ACTH response to CRH was almost three times lower after dexamethasone pretreatment (1483 $\pm$ 794 mm^2 vs 4180 $\pm$ 697 mm^2; $P > 0.10$), the integrated cortisol response to CRH was more than ten times lower after dexamethasone (300 $\pm$ 181 mm^2 vs 3222 $\pm$ 612 mm^2; $P < 0.02$).

2. Relationship between basal dexamethasone levels and CRH induced changes in ACTH and cortisol after overnight suppression with increasing doses of dexamethasone

Individual basal plasma dexamethasone levels (i.e. the dexamethasone levels immediately before CRH administration at 09.00 h after overnight suppression with dexamethasone) were the higher, the higher the dose of dexamethasone (Table 1).
Figures 2 and 3 illustrate that there was a significant and negative correlation between the basal dexamethasone levels in the experiments A to D and the areas under the individual ACTH ($r = -0.62$; $P^{**} < 0.01$) and cortisol ($r = -0.81$; $P^{**} < 0.001$) curves, i.e. the lower the basal dexamethasone level, the greater the rise in ACTH and cortisol levels after CRH administration.

DISCUSSION

In previous studies (1,2) we have shown that in healthy subjects the basal plasma cortisol level determines the response of the pituitary-adrenal axis to CRH. So far it has not been proven that the inverse correlation between the basal plasma cortisol level and the response of ACTH and cortisol to CRH in man is indeed the result of a direct feedback effect of cortisol at the level of the corticotrophs. One might wonder whether this correlation is only spurious, dictated by an, as yet undetected underlying correlation between the individual endogenous CRH levels and the responses of ACTH and cortisol to exogenous CRH.

Table 1. Pre-CRH dexamethasone levels (ng/dl) in five healthy subjects after overnight suppression with different doses of dexamethasone (DXM)

Subject no	Experiment A (1mg DXM)	Experiment B (2mg DXM)	Experiment C (4mg DXM)	Experiment D (2+2mg DXM)
1	100	207	212	946
2	210	227	755	1270
3	230	360	746	1869
4	302	331	419	1271
5	118	506	-	947
Mean±SEM	192 ± 33	326 ± 48	533 ± 115	1261 ± 150[a]

[a] $P < 0.005$ vs experiment A and C; $P < 0.01$ vs experiment B.

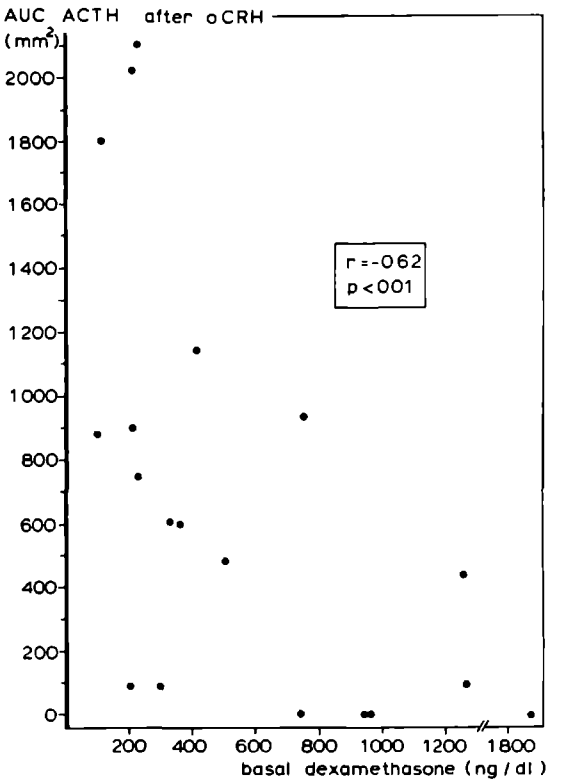

Figure 2. Intravenous bolus injection of 200 μg ovine CRH after overnight suppression with various doses of dexamethasone (experiments A to D, see text) in 5 normal subjects; relation between basal dexamethasone levels and the areas below the individual ACTH curves.

The present study demonstrates a highly significant and negative correlation between the responses of ACTH and cortisol to CRH and the levels of the synthetic glucocorticoid dexamethasone, measured immediately before the injection of CRH after overnight suppression with this steroid Studies in rodents have demonstrated that pharmacological doses of dexamethasone suppress endogenous CRH activity (3-7) Under the proviso that in man overnight treatment with pharmacological doses of dexamethasone will also suppress endogenous CRH activity, our study demonstrates that the level of circulating glucocorticoids per se determines the response to CRH in man. The observation that a single dose of 1 mg dexamethasone one hour before CRH injection leads to a significant inhibition of the CRH induced ACTH and cortisol response - despite unsuppressed basal ACTH and cortisol levels - also agrees with this conclusion

We found that ovine CRH in all individuals caused an escape from the inhibitory effects of overnight suppression with single doses of dexamethasone of up to 4 mg

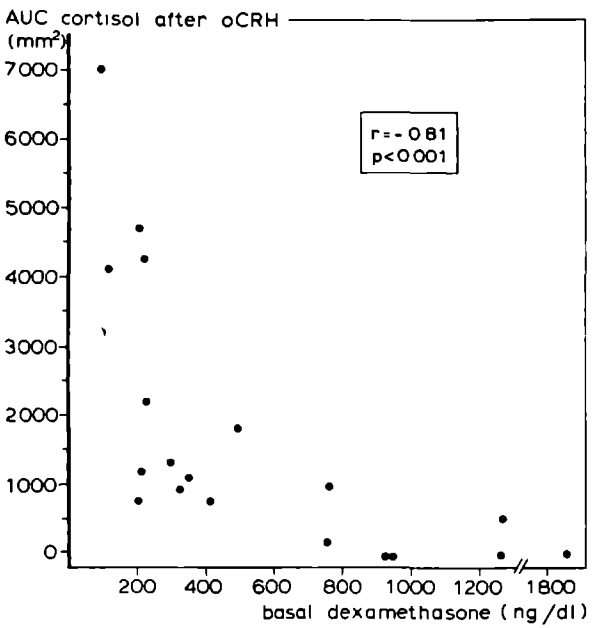

Figure 3 Intravenous bolus injection of 200 μg ovine CRH after overnight suppression with various doses of dexamethasone (experiments A to D, see text) in 5 normal subjects, relation between basal dexamethasone levels and the areas below the individual cortisol curves

This data is seemingly in contrast with that of Lytras et al (9) and Copinschi et al (10), who showed that the cortisol response to 100 and 50 μg ovine CRH respectively, is completely prevented by overnight treatment with 1/2 and 1 mg dexamethasone respectively, followed by 1/2 and 1 mg respectively, on the morning of the CRH injection. However, unpublished data from our laboratory shows that their regimes of dexamethasone treatment cause higher dexamethasone levels than overnight suppression with a single dose of 4 mg dexamethasone. These higher dexamethasone levels may account for the prevention of activation of the pituitary-adrenal axis by CRH, comparable to our results after pretreatment with 2 doses of 2 mg dexamethasone. Our data is also seemingly in contrast with a recent study of Von Bardeleben et al (11). In this study CRH alone was not able to produce a marked elevation of plasma ACTH and cortisol in healthy subjects after overnight suppression with 1,5 mg dexamethasone. It has to be stressed, however, that peak responses in all individuals in our study occurred in the third or fourth hour after CRH injection after overnight suppression with dexamethasone. Von Bardeleben and co-workers used human CRH, which has a significantly shorter duration of action than the ovine peptide used in our study, and measured ACTH and cortisol levels only during the first two hours after CRH. Moreover, in our study a twice higher dose of CRH was given. The results of our study are in line with those of De Bold et al. (12), who found a significant negative correlation between plasma dexamethasone levels and peak ACTH and cortisol levels after CRH administration after pretreatment with replacement doses (0,5 mg) of dexamethasone, given at various time-intervals before CRH injection.

It has been suggested that the absence of plasma cortisol suppression the day after an evening dose of 1 or 2 mg dexamethasone in patients with endogenous depression is caused by exaggerated CRH drive in this disorder. Our observation that ovine CRH caused an escape from the inhibitory effect of overnight suppression with dexamethasone demonstrates that excessive CRH drive is able to override the inhibitory effects of relatively high doses of dexamethasone and that this mechanism indeed can be the cause of the abnormal dexamethasone suppression test results in depressed patients.

We conclude that CRH overrules the inhibition of the pituitary-adrenal axis by physiological and pharmacological doses of dexamethasone. The strongly negative correlation between the basal dexamethasone levels and the CRH induced pituitary-adrenal response argues for the primacy of circulating glucocorticoid levels in the modulation of the response of ACTH and cortisol to CRH.

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2.5 ENHANCEMENT OF THE ACTH RESPONSE TO HUMAN CRH BY PRETREATMENT WITH THE ANTIGLUCOCORTICOID RU-486

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SUMMARY

The response of ACTH to an i.v. bolus injection of 100 μg human CRH at 09.00 h was investigated in five healthy men, with and without pretreatment with 100 mg of the antiglucocorticoid RU-486, administered orally seven hours before CRH injection. In all five subjects the plasma cortisol level immediately before CRH administration at 09.00 h was higher after pretreatment with the antiglucocorticoid ($17{,}1 \pm 2{,}1$ vs $11{,}1 \pm 1{,}1$ μg/100 ml; $P < 0.05$). Despite this higher baseline cortisol level, in all subjects the maximal CRH induced ACTH increase was more pronounced after RU-486 loading ($_{\Delta}$ max ACTH 39 ± 14 vs 26 ± 13 pg/ml; $P < 0.05$). This observation proves that ,in man, physiological concentrations of cortisol determine the response of the pituitary to CRH. Furthermore, these findings suggest deficient circulating glucocorticoid activity after the administration of 100 mg RU-486, not completely compensated for by the increase of plasma cortisol levels after RU-486.

INTRODUCTION

In previous studies (1,2) we have shown that the response of the pituitary-adrenal axis to CRH in healthy man is modulated by the individual basal cortisol level, i.e. CRH administration results in the lesser stimulation of the pituitary-adrenal axis when basal cortisol levels are the higher. This observation is compatible with, but does not prove, the concept that the level of circulating cortisol itself determines the response of the pituitary to CRH in man (1).

Submitted for publication

The recently developed steroid RU-486 (17β-hydroxy-11β-[4-di-methyl-aminophenyl]17α-[1-propynyl] estra-4,9-dien-3-one) is the first glucocorticoid antagonist active in vivo in rodents (3), nonhuman primates (4,5) and man (6).

If it is indeed true that physiological concentrations of cortisol inhibit the response of the pituitary to CRH, blockade of this negative feedback action on the corticotrophs by using sufficient amounts of a competitive antagonist should increase the response of ACTH to CRH. Therefore we investigated whether after pretreatment with the antiglucocorticoid RU-486 the CRH induced ACTH increase in man is enhanced.

SUBJECTS AND METHODS

Five healthy men (aged 22 ± 3 years, mean ± SD) volunteered in this study after approval of the protocol by the hospital ethical committee. The five subjects participated in two experiments. In both experiments 100 μg human CRH (Bachem, Torrance, CA) was administered as an i.v. bolus injection at 09.00 h (t = 0 min), either without (experiment A) or with (experiment B) oral pretreatment with 100 mg RU-486 (Institut Roussel Uclaf, Paris, France) seven hours before the injection of CRH. Both experiments were performed on separate occasions with an interval of at least three days. Three subjects took experiment A first, and the remaining two subjects experiment B.

CRH was given after an overnight fast with the subjects supine since 08.00 h. The peptide was dissolved in 1 ml acid-saline (pH 2) immediately before administration. Blood samples for plasma ACTH and cortisol assay were collected at -30, 0, 5, 10, 20, 30, 60, 120, 180 and 240 min through an indwelling i.v. catheter kept open with small amounts of a diluted (10%) solution of heparin. The catheter was inserted one hour before CRH administration. Plasma ACTH and cortisol were measured by specific RIA's as described previously (7).

Statistical analysis was performed using Student's t-test for paired observations. The mean values ± 1 SEM are given.

RESULTS (Figure 1, Table 1)

Figure 1 shows that RU-486 pretreatment significantly increased the mean plasma ACTH and cortisol levels both before and after CRH administration. Table 1 illustrates that in all five subjects the basal (t = 0 min) cortisol level was higher after pretreatment with the antiglucocorticoid (17,1 ± 2,1 vs 11,1 ± 1,1 μg/100 ml; $P < 0.05$). Despite this higher baseline cortisol level, in all subjects the maximal CRH induced ACTH increase was more

pronounced after RU-486 loading (Δ max ACTH 39 ± 14 vs 26 ± 13 pg/ml; $P < 0.05$; table 1).

DISCUSSION

Pharmacological doses of glucocorticoids inhibit the pituitary-adrenal axis in man. In addition, it has been demonstrated that physiological concentrations of circulating corticosteroids suppress the basal and/or stimulated ACTH secretion in rodents (8, 9), dogs (10-12) and ovine fetuses (13-15). However, the physiological significance of corticosteroid-mediated negative feedback *in man* is still a matter of debate (16).

The present study shows that a single oral dose of 100 mg of the antiglucocorticoid RU-486 at 02.00 h leads to a significant increase in the mean ACTH and cortisol level six to seven hours later. Most probably the increase of ACTH and cortisol after RU-486 is caused by blockade of the negative feedback action of circulating cortisol at the pituitary and/or hypothalamic level. This demonstrates that physiological concentrations of cortisol can inhibit the hypothalamic-pituitary-adrenal system in man. In addition we demonstrate that loading with the antihormone significantly increases the mean maximal CRH induced ACTH rise, despite a significantly higher circulating basal cortisol level. In our opinion this observation provides compelling evidence that in man physiological concentrations of cortisol also determine the response of the pituitary to CRH.

As a final comment, this study may be of clinical interest, because RU-486 is not only an antagonist of cortisol, but also of progesterone and as such is in

Table 1 Individual basal plasma cortisol levels (i.e plasma cortisol levels immediately before CRH administration) and maximal ACTH increases after an i v bolus injection of 100 μg human CRH in five men with and without oral pretreatment with 100 mg RU-486 seven hours before the injection of CRH

	basal cortisol (μg/100 ml)		Δ max ACTH after 100 μg hCRH (pg/ml)	
Subject no.	control	on RU-486	control	on RU-486
1	7,6	10,5	79	99
2	12,3	24,6	11	23
3	14,8	16,3	5	9
4	10,5	19,5	28	31
5	10,1	14,8	7	33
Mean ± SEM	11,1 ± 1,1[a]	17,1 ± 2,1	26 ± 13[a]	39 ± 14

a) $P < 0.05$ vs on RU-486

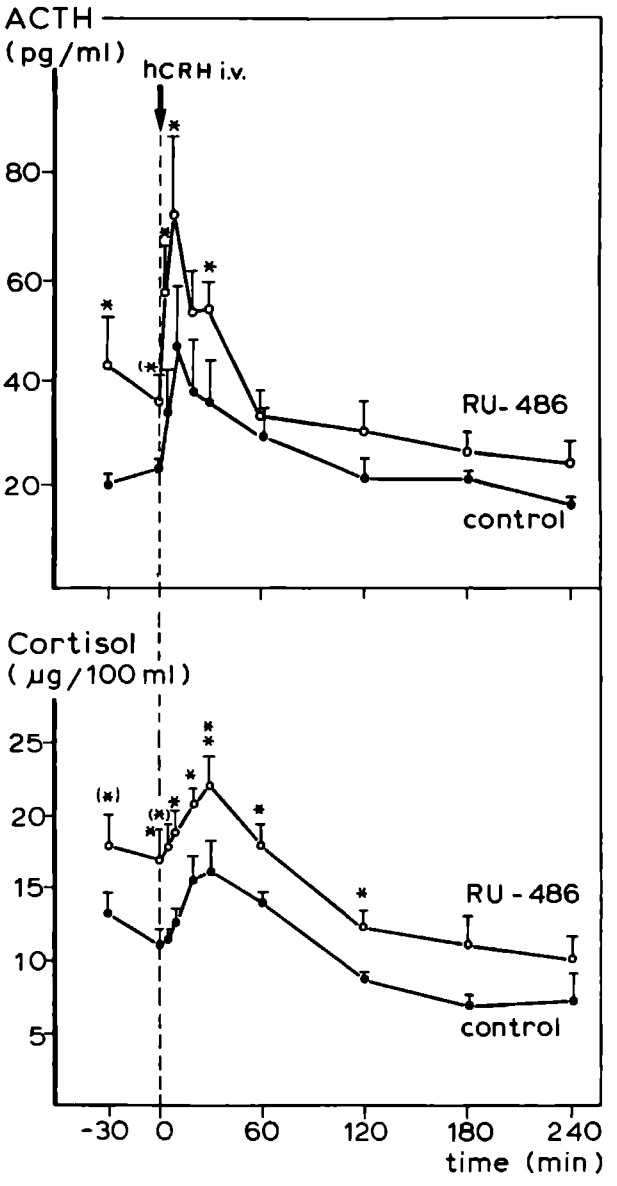

Figure 1. Mean ± SEM plasma ACTH and cortisol levels before and after an i.v. bolus injection of 100 µg human CRH in five healthy men:

● without pretreatment with RU-486

○ after oral pretreatment with 100 mg RU-486 seven hours before the injection of CRH.

Asterisks indicate statistical significance between RU-486 and control (*) $P < 0.10$; * $P < 0.05$; ‡ $P < 0.01$.

use as a contragestive agent (17). The ACTH response to exogenously administered CRH has been recommended as a bio-assay for circulating glucocorticoid activity (18), i.e. the lesser the circulating glucocorticoid activity, the higher the ACTH response to CRH. We observed that a single dose of 100 mg RU-486 significantly enhances the ACTH response of the pituitary to CRH. We interpret this as an indication of deficient glucocorticoid activity after RU-486 administration, not fully compensated for by the increased levels of plasma cortisol after administration of the antiglucocorticoid.

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Chapter 3

Studies in patients with disorders of the hypothalamic-pituitary-adrenal axis

3.1 RESPONSIVITY OF ACTH TO CRH AND LACK OF SUPPRESSIBILITY BY DEXAMETHASONE ARE RELATED PHENOMENA IN CUSHING'S DISEASE*

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SUMMARY

The ACTH and cortisol responses to 100 μg ovine CRH in 19 consecutive patients with Cushing's disease were compared with those in 13 normal subjects. In two patients with Cushing's disease plasma ACTH and cortisol did not increase after CRH administration. As compared to the normal subjects maximal ACTH increments (135 $\pm$ 25 (SEM) vs 42 $\pm$ 6 pg/ml; P* $<$ 0,001) and maximal cortisol increments (17,7 $\pm$ 1,8 vs 9,4 $\pm$ 1,1 μg/100 ml; P* $<$ 0,01) after CRH were significantly higher in the 17 CRH-responsive patients with Cushing's disease. In the normal subjects there was a significant negative correlation between the basal cortisol level and the maximal ACTH (r = -0,65, P** $<$ 0,05) and cortisol (r = -0,95, P** $<$ 0,001) responses to CRH. In contrast, in the CRH-responsive Cushing patients there was no significant correlation between the basal cortisol level and the maximal ACTH (r = +0,10, P** $>$ 0,10) and cortisol (r = +0,14; P** $>$ 0,10) increments after CRH. In the normal subjects there was no significant correlation between the basal ACTH level and the maximal ACTH increments after CRH (r = -0,24, P** $>$ 0,10). Again in contrast, in the CRH-responsive Cushing patients maximal ACTH increments after CRH correlated positively with the basal ACTH levels (r = +0,69, P** $<$ 0,005). Moreover, in these patients the maximal ACTH increments after CRH were positively correlated with the ACTH levels after suppression with higher and lower doses of dexamethasone. We conclude that in Cushing's disease - unlike in normal subjects -circulating cortisol is not the major modulator of the ACTH and cortisol response to CRH and that in these patients

* Presented in part at the 67th Annual Meeting of the Endocrine Society (Abstract 1004), Baltimore, June 19-21, 1985.

responsivity of ACTH to CRH and lack of suppressibility by dexamethasone are related phenomena.

INTRODUCTION

We previously demonstrated that in normal subjects the ACTH and cortisol responses to ovine or human corticotropin-releasing hormone (CRH) depend on the baseline cortisol level, i.e. the higher the baseline cortisol level, the lesser the response to CRH (1,2). Most patients with pituitary-dependent Cushing's disease have ACTH responses to CRH (3-7), although the individual ACTH and cortisol increases after CRH are highly variable, and small (8) or even absent responses (4, 9-11) to CRH have been reported. So far, the factors determining pituitary-adrenal responsiveness to CRH in patients with Cushing's disease have not been evaluated. Therefore, we compared the ACTH and cortisol responses to CRH to the basal ACTH and cortisol levels and their responses to dexamethasone in 19 such patients.

METHODS

Patients

Pituitary-adrenal responsiveness to ovine CRH was studied in 19 consecutive patients with pituitary-dependent Cushing's disease. The study protocol was approved by the hospital ethical committee and informed consent was obtained from all subjects. The results in the patients were compared with data from 13 normal subjects (8 men, aged 24 ± 4 years (mean ± SD) and 5 women, aged 31 ± 14 years). The clinical data of the patients are given in Table 1. All female patients except numbers 7, 17 and 19 were premenopausal. None of the patients had visual field defects and none had sellar destruction, except patient 17, who had a macroadenoma. Fourteen patients were studied before they received any therapy. The other five patients (1, 4, 7, 14 and 17) had previously (i.e. before they were tested with CRH) undergone pituitary surgery, which was unsuccessful despite partial removal of a pituitary adenoma in patients 4, 7 and 17. These 5 patients, except patient 4, were cured after participating in this study by bilateral adrenalectomy. Patients 6, 8, 9, 11, 12, 13 and 15 were cured later by transsphenoidal or transfrontal removal of a pituitary adenoma. Patients 10 and 19 were treated by bilateral adrenalectomy. Patients 2, 3, 5, 16 and 18 were not cured after transsphenoidal operation, despite partial removal of a pituitary adenoma in 2, 3 and 5. These 5 patients then were cured by bilateral adrenalectomy. Table 2 summarizes the treatment modalities carried out in the 19 patients after the CRH test, as well as the results of histological examination of tissue removed during pituitary or adrenal surgery.

Table 1 Clinical data of 19 consecutive patients with Cushing's disease

Patient no	Age (years)	Sex	Duration of disease (years)	Basal plasma ACTH[a] (pg/ml)	Basal plasma cortisol[b] (μg/100ml)	Basal urinary 17 OHCS (μmol/mmol creatinine)[c]	17 OHCS(μmol/mmol creatinine) after 2 mg dexamethasone q.i.d. for 2 days[d]
1	17	M	6	103	23,5	9,3	0,7
2	25	F	1,5	90	22,4	7,5	0,3
3	51	F	1	126	38,7	40,0	21,9
4	39	M	3	104	14,1	4,6	0,7
5	24	F	3	117	26,1	9,2	0,4
6	34	F	3	53	27,5	8,2	1,7
7	43	F	2	70	11,2	-	-
8	38	M	5	49	15,2	14,0	0,5
9	32	F	1	62	21,7	6,2	0,9
10	25	F	4	30	19,5	4,8	0,7
11	24	F	2	39	21,0	7,5	0,8
12	20	F	2	51	21,0	6,9	0,3
13	53	M	1	85	27,2	11,1	0,6
14	18	F	4	54	20,6	4,2	-
15	35	F	0,5	140	24,3	8,7	3,1
16	54	F	2	77	16,7	8,5	0,1
17	49	F	6	151	12,7	-	-
18	37	M	2,5	71	20,6	10,4	-
19	55	F	2	31	16,7	11,3	9,1

a Basal plasma ACTH represents the mean of 4 samples taken at 9 a m. (normal value < 75 pg/ml).

b Basal plasma cortisol represents the mean of 4 samples taken at 9 a m. (normal range 6,9 -19,9 μg/100ml).

c Normal range 0,6 - 3,0 μmol/mmol creatinine (1 μmol/mmol creatinine = 2,48 mg/g creatinine)

d In four patients 17 OHCS excretion was not determined. Plasma cortisol levels after 2 mg dexamethasone q.i.d. for 2 days were 0,7 μg/100 ml in patient 7, 9,1 μg/100 ml in patient 14, and 8,7 μg/100 ml in patient 18.

Table 2 Treatment modalities of the patients *after* the CRH- and dexamethasone-tests Results of histological examinations

Patient no	Treatment[a]	Histological examination of tissue removed during pituitary surgery	Histological examination of both removed adrenals[b]	Adrenal weight (gr)	
1	ADX	-	diffuse hyperplasia	R 4,2	L 5,0
2	TS, ADX	adenoma	diffuse hyperplasia	R 7,0	L 7,0
3	TS, ADX	adenoma	diffuse hyperplasia	R 7,5	L 9,0
4	No further treatment	-	-	-	
5	TS, ADX	adenoma	diffuse hyperplasia	R 5,7	L 6,1
6	TS	adenoma	-	-	
7	ADX	-	macronodular hyperplasia (R 2,2 cm, L 3,5 cm)	R 15,5	L 37,3
8	TS	adenoma	-	-	
9	TS	adenoma	-	-	
10	ADX	-	diffuse hyperplasia	R 9,5	L 9,0
11	TF	adenoma	-	-	
12	TS	adenoma	-	-	
13	TF	adenoma	-	-	
14	ADX	-	diffuse hyperplasia	R 4,0	L 5,1
15	TS	adenoma	-	-	
16	TS, ADX	no adenoma found	macronodular hyperplasia (R 3,5 cm, L 1,0 cm)	R 13,9	L 6,7
17	ADX	-	diffuse hyperplasia	R 7,3	L 7,5
18	TS, ADX	no adenoma found	macronodular hyperplasia (R 0,4cm, L 1,0cm)	R 7,1	L 10,3
19	ADX	-	macronodular hyperplasia (R 5,5 cm, L 4,0cm)	R 43,0	L 44,5

a ADX = bilateral adrenalectomy, TS = transsphenoidal surgery, TF = transfrontal surgery

b maximal size of nodules in each gland (R = right, L = left) is indicated in parentheses

CRH test

Ovine CRH was obtained from Bachem (Torrance, CA). It was dissolved in a 2% aqueous solution of lactose and sterilized by passage through a 0,2 μm cellulose acetate filter (Schleicher und Schuell, FPO 30/3) and was found to be nonpyrogenic ("Pyrogent test", Mallinckrodt). CRH was stored at -18°C in lyophilized form in sterile vials under vacuum. Immediately before administration it was dissolved in 1 ml acidified NaCl (pH 2). 100 μg ovine CRH was given at 09.00 h as an i.v. bolus injection over 30 sec. with the subjects fasting and supine since 08.00 h. The cannula for blood drawing was inserted at this time. Blood samples for ACTH and cortisol assay were collected at -30, 0, 5, 10, 20, 30, 60, 120 and 180 min via an indwelling i.v. cannula kept open with a diluted (10%) solution of heparin. Plasma ACTH and cortisol were determined by specific RIAs as described previously (12). To avoid interassay variations all samples of individual patients were analyzed in the same assay.

Dexamethasone suppression tests

Dexamethasone suppression tests were performed in the patients with Cushing's disease using 2 mg dexamethasone overnight, 0,5 mg dexamethasone q.i.d. for 2 days, 2 mg dexamethasone q.i.d. for 2 days, and 4 mg dexamethasone q.i.d. for 2 days. 24 h urinary excretion of 17-hydrocorticosteroids (17 OHCS) was measured using the method described by Van De Calseyde et al (13).

Statistics

Statistical analyses were performed using Wilcoxon's paired rank test (P values denoted by P), Wilcoxon's two sample test (P = P*) and Spearman's rank correlation test (P = P**). The mean values ± 1 SEM are given.

RESULTS (Figure 1-4, Table 3)

Responses of ACTH and cortisol to CRH in patients with Cushing's disease and in normal subjects

Figures 1 and 2 illustrate the relation between the basal cortisol levels and the maximal ACTH and cortisol responses to the administration of 100 μg ovine CRH in the 19 patients with Cushing's disease as well as in the 13 normal subjects. In patients 18 and 19 (Tables 1-2) plasma ACTH and cortisol did not increase after CRH injection on two separate occasions. In the remaining 17 Cushing patients (indicated below as "CRH-responders"), mean plasma

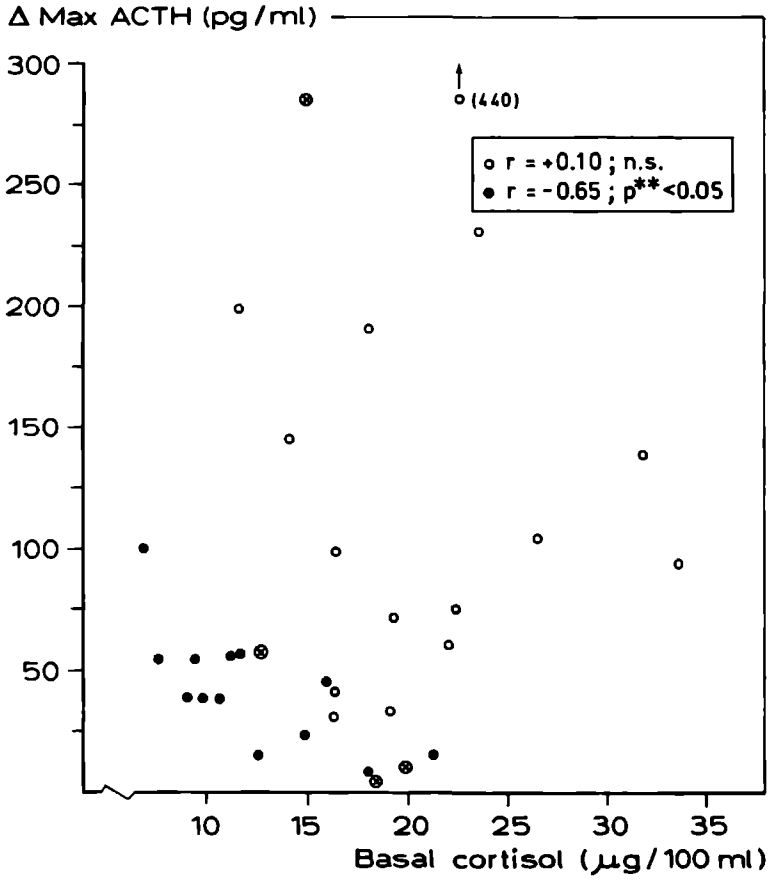

Figure 1. The relation between individual basal plasma cortisol levels and the maximal plasma ACTH increases after CRH ($=_{\Delta}$ max ACTH) in 13 normal subjects (closed symbols) and in 19 patients with Cushing's disease (open symbols).
⊗ denotes patients with macronodular hyperplasia.

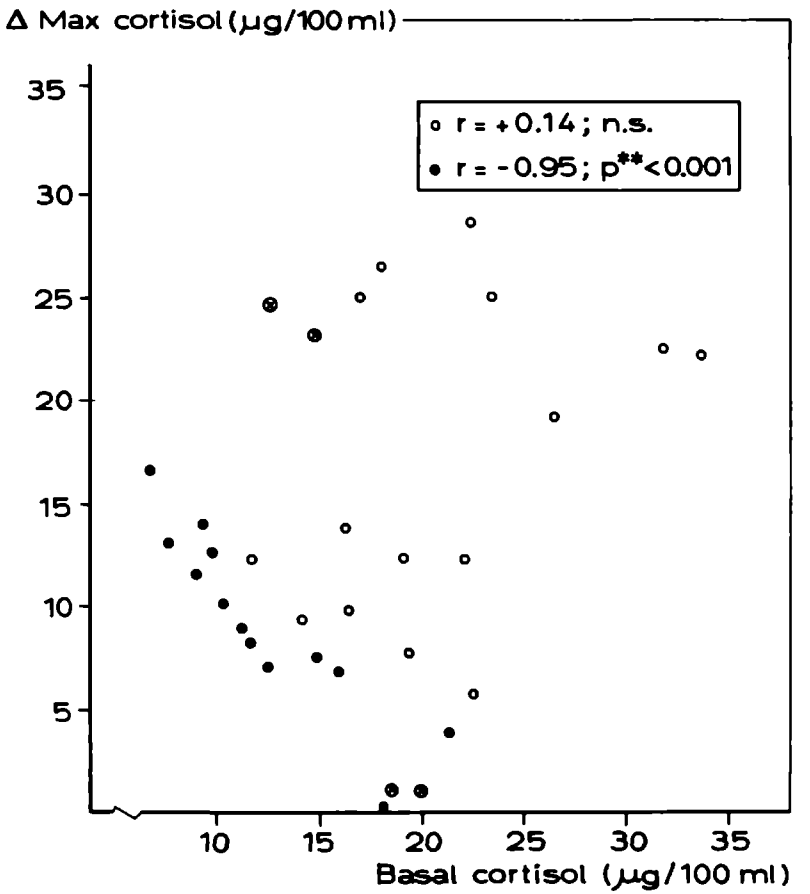

Figure 2. The relation between individual basal plasma cortisol levels and the maximal plasma cortisol increases after CRH (= $_{\Delta}$ max cortisol) in 13 normal subjects (closed symbols) and in 19 patients with Cushing's disease (open symbols).
⊗ denotes patients with macronodular hyperplasia.

ACTH increased after CRH from a basal value of 85 ± 10 pg/ml to a peak value of 197 ± 27 pg/ml at 20 min ($P < 0,01$ vs t = 0 min). Thereafter ACTH levels declined significantly to 90 ± 15 pg/ml at 180 min ($P < 0,01$ vs t = 20 min, $P > 0,10$ vs t = 0 min). Individual peak levels occurred between 5 and 120 min. In all CRH-responders plasma ACTH rises were followed by marked increases of cortisol from a baseline value of 20,3 ± 1,4 μg/dl to a peak value of 34,4 ± 3,3 μg/dl at 60 min ($P < 0,01$ vs t = 0 min). Thereafter cortisol levels declined to 27,2 ± 2,2 μg/dl at 180 min ($0,05 < P < 0,10$ vs t = 60 min; $P < 0,01$ vs t = 0 min). Individual peak levels were reached between 20 and 180 min.

Both in the normal subjects ($r = +0,70$; $P^{**} < 0,02$) and in the CRH-responding Cushing patients ($r = +0,50$; $P^{**} < 0,05$) there was a significant and positive correlation between the maximal ACTH and cortisol responses to CRH.

Table 3 illustrates that mean plasma ACTH and cortisol levels both before and after CRH administration were significantly higher in the CRH-responsive Cushing patients than in the normal subjects and that in terms of absolute increments these Cushing patients were hyperresponsive to CRH.

Analysis of factors determining the ACTH and cortisol responses in normal subjects and in CRH-responsive Cushing patients

In the 13 normal subjects the maximal ACTH responses to CRH were significantly and negatively correlated with the basal cortisol levels ($r = -0,65$; $P^{**} < 0,05$) (Fig. 1). An even stronger correlation was found between the maximal cortisol responses to CRH and the basal cortisol levels ($r = -0,95$; $P^{**} < 0,001$) (Fig. 2). In the normal subjects there was no significant correlation between basal ACTH levels and maximal ACTH ($r = -0,24$; $P^{**} > 0,10$) and maximal cortisol ($r = -0,40$; $P^{**} > 0,10$) increments after CRH. In contrast to the normal subjects there was no correlation between the basal cortisol level and the maximal ACTH ($r = +0,10$; $P^{**} > 0,10$) and maximal cortisol ($r = +0,14$; $P^{**} > 0,10$) increments after CRH in the CRH-responsive Cushing patients (Fig. 1 and 2). Again in contrast to the normal subjects, a highly significant positive correlation was found between the basal ACTH levels and the maximal ACTH increments after CRH ($r = +0,69$; $P^{**} < 0,005$) (Fig. 3). There was no significant correlation between basal ACTH and the maximal cortisol response after CRH ($r = +0,25$; $P^{**} > 0,10$).

The maximal ACTH increments correlated not only with the basal ACTH levels, but also with the ACTH levels after 2 mg dexamethasone overnight (62 ± 8 pg/ml; $r = +0,59$; $P^{**} < 0,05$; $n = 12$), the ACTH levels after 4 x 0,5 mg dexamethasone for 2 days (61 ± 9 pg/ml; $r = +0,49$; $P^{**} < 0,05$; $n = 16$) and the ACTH levels after 4 x 4 mg dexamethasone for 2 days (49 ± 15

Table 3 Comparison of basal plasma ACTH and cortisol levels and responses of these hormones to 100 μg oCRH in 13 normal subjects and 17 CRH-responding patients with Cushing's disease

	Healthy subjects	Patients with Cushing's disease
basal ACTH (pg/ml)	25 ± 2*	85 ± 10[c]
maximal ACTH increment after CRH	42 ± 6	135 ± 25[b]
peak ACTH after CRH**	67 ± 6	220 ± 32[c]
basal cortisol (μg/dl)	12,3 ± 1,1	20,3 ± 1,4[b]
maximal cortisol increment after CRH	9,4 ± 1,1	17,7 ± 1,8[a]
peak cortisol after CRH**	21,7 ± 0,7	37,6 ± 2,5[d]

* mean ± SEM

** the means of individual peaks, independent of time are given

([a], $P^* < 0.01$ vs normal subjects;
[b], $P^* < 0.001$,
[c], $P^* < 0.0001$;
[d], $P^* < 0.00001$)

pg/ml; r = +0,73; P** < 0,005; n = 13) (Fig.4). The correlation between the maximal ACTH responses after CRH and the ACTH levels after 4 x 2 mg dexamethasone (51 ± 9 pg/ml; n = 15) was not significant (r = +0,36; P** > 0,10).

DISCUSSION

We analyzed the ACTH and cortisol responses to 100 μg ovine CRH in 19 consecutive patients with pituitary-dependent Cushing's disease and compared the responses with those in 13 normal subjects. Moreover, in the 17 CRH-responding Cushing patients ACTH responses to CRH were compared with the suppressibility of ACTH levels by dexamethasone.

As we previously demonstrated (1,2), in the normal subjects the ACTH and cortisol responses to CRH were modified to a high degree by the basal level of circulating cortisol, i.e. the higher the basal cortisol, the lower the response to CRH. The basal ACTH levels, however, did not correlate significantly with the response to CRH.

In the 17 CRH-responsive patients with Cushing's disease, individual ACTH and cortisol profiles after CRH were highly variable. Compared to the individual responses in the normal subjects, taking into account the basal

cortisol levels, a normal ACTH response to CRH was found in four and a normal cortisol response in five patients with Cushing's disease. In only one of the 17 patients, however, were the responses of both ACTH and cortisol to CRH within the range of those in the normal subjects.

In contrast to the normal subjects, in the CRH-responsive patients with Cushing's disease there was no correlation at all between the basal cortisol level and the ACTH and cortisol responses to CRH, confirming preliminary data of Orth et al (14). Remarkably, in the CRH-responding Cushing patients the ACTH response to CRH was highly positively correlated with the basal ACTH level. The second new finding of interest was that the response to CRH, if present, correlated positively with the plasma ACTH value after dexamethasone administration, i.e. greater responses to CRH occurred in patients whose plasma ACTH declines the least after dexamethasone.

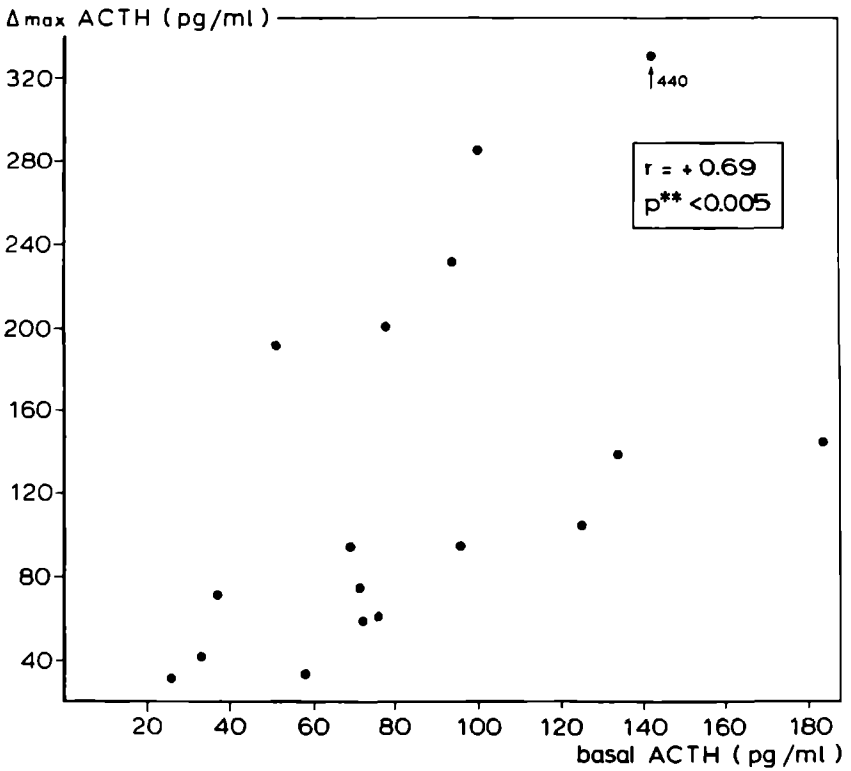

Figure 3 The relation between individual basal plasma ACTH levels and the maximal plasma ACTH increases after CRH (= Δ max ACTH) in 17 CRH-responding patients with Cushing's disease

Lack of normal suppressibility of ACTH-release by glucocorticoids is at the base of the pathophysiology in the great majority of patients with Cushing's disease. This defect of negative feedback can explain the lack of correlation between basal cortisol and CRH-induced responses of ACTH and cortisol in Cushing's disease. Our observation that in Cushing's disease the degree of lack of suppressibility of ACTH by glucocorticoids was significantly correlated with the degree of ACTH responsiveness to CRH is intriguing. Very recently it has been demonstrated that there is heterogeneity among corticotrophs of the normal rat anterior pituitary (15) and that the adenohypophysis contains a subpopulation of corticotrophs preferentially stimulated by CRH and inhibited by glucocorticoids (16). We speculate that in Cushing's disease these corticotrophs are those that proliferate and at least in part lose their ability to be inhibited by glucocorticoids without losing their sensitivity to CRH stimulation, leading to hypersecretion of ACTH. In this concept of the pathogenesis of Cushing's disease, the ACTH level after dexamethasone administration is an index of the number of these CRH-responsive and glucocorticoid insensitive cells. Then, it is not surprising that, as we found, responsivity to CRH in Cushing's disease and lack of suppressibility by dexamethasone are related phenomena.

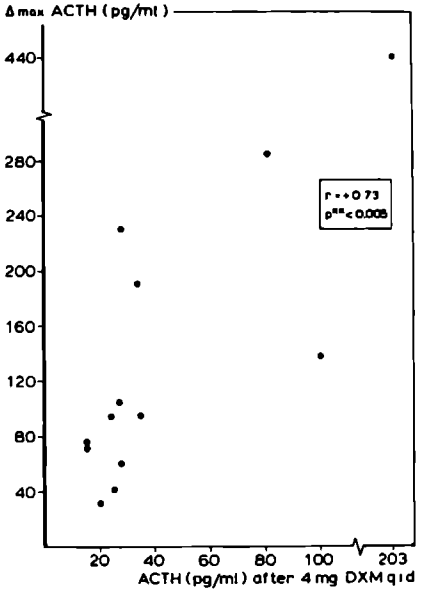

Figure 4 The relation between individual plasma ACTH levels after administration of 4 mg dexamethasone q.i.d. for 2 days and the maximal plasma ACTH increases after 100 μg CRH (= Δ max ACTH) in 13 CRH-responding patients with Cushing's disease.

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3.2 THE CRH TEST VERSUS THE "HIGH-DOSE" DEXAMETHASONE TEST IN THE DIFFERENTIAL-DIAGNOSIS OF CUSHING'S SYNDROME

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ABSTRACT

The diagnostic accuracy of the CRH test was compared with that of the oral "high-dose" dexamethasone suppression test according to Liddle in the differential-diagnosis of Cushing's syndrome. A false-negative response to CRH was present in 9% (2 of 22) of patients with pituitary-dependent Cushing's disease and to "high-dose" dexamethasone in 11% (2 of 18). All three patients with Cushing's syndrome due to an adrenal adenoma were unresponsive to both CRH and dexamethasone. The only patient with ectopic ACTH secretion in this series had a false-positive response of ACTH to dexamethasone and no response of ACTH to CRH. Simultaneous failure of both tests to indicate the cause of Cushing's syndrome did not occur in this series, except in one patient with Cushing's disease and overt macronodular hyperplasia. We conclude that the diagnostic accuracy of the CRH test in the work-up of the patient with Cushing's syndrome is comparable to that of the Liddle test and that the highest discriminatory score in the differential-diagnosis of Cushing's syndrome is achieved by performing both a CRH test and a "high-dose" dexamethasone test.

INTRODUCTION

Cushing's syndrome is caused by primary overproduction of ACTH, either by the pituitary gland or by an ectopic source, or by primary overproduction of cortisol due to an adrenal adenoma or carcinoma. Various diagnostic manoeuvres are in use to discriminate between these forms of Cushing's syndrome. However, not infrequently they lead to contradictory results, necessitating prolonged hospital stays.

Submitted for publication

After the isolation of the 41 amino acid peptide corticotropin-releasing hormone (CRH) from ovine hypothalami by Vale et al in 1981 (1) several groups have assessed the value of this peptide in the differential-diagnosis of Cushing's syndrome (2-4). Müller et al (2) and Chrousos et al (3) demonstrated that after administration of ovine CRH plasma ACTH levels in patients with pituitary-dependent Cushing's disease rose without exception. Patients with an adrenal adenoma or untreated adrenal carcinoma were characterized by undetectable baseline ACTH levels unresponsive to CRH, while patients with ectopic ACTH secretion had increased basal ACTH levels, also unresponsive to CRH. This data suggests a very high specificity of the CRH test.

So far, no studies have systematically compared the value of the CRH test with that of more conventional tests in the differential-diagnosis of Cushing's syndrome. The present study collates the diagnostic accuracy of the CRH test with that of the oral "high-dose" dexamethasone suppression test according to Liddle (5,6) in a group of 26 consecutive patients with Cushing's syndrome. In addition, we studied whether the diagnostic power of these tests combined is greater than that of each test alone.

METHODS

Patients (Table 1 and 2)

Twenty-five consecutive patients with active Cushing's syndrome (22 with pituitary-dependent Cushing's disease and three with Cushing's syndrome due to an adrenal adenoma) participated in this study. In addition, ACTH responsiveness to ovine CRH and dexamethasone was determined in one patient with ectopic ACTH secretion due to a bronchial carcinoid. The study protocol was approved by the hospital ethical committee and informed consent was obtained from all patients. Relevant clinical and biochemical data of the 25 patients with active Cushing's syndrome is given in Table 1. All female patients except numbers 7, 17 and 22 were premenopausal. None of the patients had visual field defects. In only one patient (number 17, Table 1) CT scanning demonstrated a macroadenoma. Nineteen patients were studied before they received any therapy. Five patients (1, 4, 7, 14 and 17) had previously (i.e. before entering in this study) undergone pituitary surgery, which was unsuccessful despite partial removal of a pituitary adenoma in patients 4, 7 and 17. These five patients, except patient 4, were cured after participating in this study by bilateral adrenalectomy. In patient 20 a pituitary microadenoma was removed 4,5 years earlier. Postoperatively all clinical signs and symptoms of Cushing's disease as well as hypercortisolism disappeared. In this patient, who was never hypocortisolemic after surgery, signs and symptoms of Cushing's disease recurred after a

clinical remission of more than 4 years. After completion of this study patients 6, 8, 9, 11, 12, 13, 15, 18 and 19 were treated by transsphenoidal or transfrontal removal of a pituitary adenoma. Patients 10, 20 and 22 were treated by bilateral adrenalectomy. In patients 2, 3, 5, 16 and 21 Cushing's disease was still active after transsphenoidal operation, despite partial removal of a pituitary adenoma in 2, 3 and 5. These 5 patients were then given relief by bilateral adrenalectomy. The patients with Cushing's syndrome due to an adrenal adenoma were treated by unilateral adrenalectomy. Table 2 summarizes the treatment modalities carried out in the 25 patients with active Cushing's syndrome after the CRH test and the "high-

Table 1. Clinical and biochemical data of 22 consecutive patients with Cushing's disease (patients 1-22) and of 3 patients with Cushing's syndrome due to an adrenal adenoma (patients 23-25)

Patient no	Age (yrs)	Sex	Duration of disease (yrs)	Basal ACTH[a] (pg/ml)	Basal cortisol[b] (μg/100ml)
1	17	M	6	103	23,5
2	25	F	1,5	90	22,4
3	51	F	1	126	38,7
4	39	M	3	104	14,1
5	24	F	3	117	26,1
6	34	F	3	53	27,5
7	43	F	2	70	11,2
8	38	M	5	49	15,2
9	32	F	1	62	21,7
10	25	F	4	30	19,5
11	24	F	2	39	21,0
12	20	F	2	51	21,0
13	53	M	1	85	27,2
14	18	F	4	54	20,6
15	35	F	0,5	140	24,3
16	54	F	2	77	16,7
17	49	F	6	151	12,7
18	40	M	1	35	18,5
19	24	F	0,5	37	27,2
20	41	F	0,25	53	19,2
21	37	M	2,5	71	20,6
22	55	F	2	31	16,7
23	39	F	2	< 15	20,6
24	20	F	3	< 15	20,6
25	30	F	2,5	< 15	19,5

a basal ACTH represents the mean of 4 samples taken at 9 a.m. (normal value < 75 pg/ml)

b basal cortisol represents the mean of 4 samples taken at 9 a.m. (normal range 6,9 - 19,9 μg/100ml)

Table 2 Treatment modalities of the 22 patients with Cushing's disease (1-22) and the three patients with Cushing's syndrome due to an adrenal adenoma (23-25) after the CRH test Results of histological examinations

Patient no	Treatment[a]	Histological examination of tissue removed during pituitary surgery	Histological examination of removed adrenal(s)[b]	Adrenal weight (gr)	
1	ADX	-	diffuse hyperplasia	R 4,2	L 5,0
2	TS, ADX	adenoma	diffuse hyperplasia	R 7,0	L 7,0
3	TS, ADX	adenoma	diffuse hyperplasia	R 7,5	L 9,0
4	No further treatment	-	-	-	
5	TS, ADX	adenoma	diffuse hyperplasia	R 5,7	L 6,1
6	TS	adenoma	-	-	
7	ADX	-	macronodular hyperplasia (R 2,2 cm, L 3,5 cm)	R 15,5	L 37,3
8	TS	adenoma	-	-	
9	TS	adenoma	-	-	
10	ADX	-	diffuse hyperplasia	R 9,5	L 9,0
11	TF	adenoma	-	-	
12	TS	adenoma	-	-	
13	TF	adenoma	-	-	
14	ADX	-	diffuse hyperplasia	R 4,0	L 5,1
15	TS	adenoma	-	-	
16	TS, ADX	no adenoma found	macronodular hyperplasia (R 3,5 cm, L 1,0 cm)	R 13,9	L 6,7
17	ADX	-	diffuse hyperplasia	R 7,3	L 7,5
18	TS	adenoma	-	-	
19	TS	adenoma	-	-	
20	ADX	-	diffuse hyperplasia	R 6,7	L 8,3
21	TS, ADX	no adenoma found	macronodular hyperplasia (R 0,4 cm, L 1,0 cm)	R 7,1	L 10,3
22	ADX	-	macronodular hyperplasia (R 5,5 cm, L 4,0 cm)	R 43,0	L 44,5
23	left adrenalectomy	-	adrenal adenoma		L 7,2
24	right adrenalectomy	-	adrenal adenoma	R 13,0	
25	left adrenalectomy	-	adrenal adenoma		L 11,0

a ADX = bilateral adrenalectomy, TS = transsphenoidal surgery, TF = transfrontal surgery

b in patients with bilateral macronodular hyperplasia maximal size of nodules in each gland (R = right, L = left) is indicated in parentheses

dose" dexamethasone test, as well as the results of the histological examination of material removed during pituitary or adrenal surgery.

The woman with ectopic ACTH secretion was 60 years old when severe Cushing's syndrome was diagnosed in 1978. Because of hyperpigmentation and an unusually high plasma ACTH level (700 pg/ml) the possibility of ectopic ACTH secretion was considered, but in spite of extensive investigations (including lung planigrams) no tumor could be detected and therefore a tentative diagnosis of pituitary-dependent Cushing's disease was made, mainly because of suppression of plasma cortisol to 30% of the basal value after treatment with 8 mg dexamethasone for 2 days. She was treated by bilateral adrenalectomy and was well until September 1984, when she complained of dyspnea and wheezing. A carcinoid tumor with obstruction of the right upper bronchus and metastases to mediastinal lymph nodes and to the liver was diagnosed. Immunohistochemical examination of the bronchial carcinoid (Dr.A.C.Nieuwenhuyzen Kruseman, University Hospital, Leiden) revealed that the tumor cells contained ACTH, β-endorphin and γ-MSH, but no CRH.

CRH test

The CRH test was performed at 09.00 h with the subjects fasting and at bed rest. Ovine CRH (100 μg: Bachem, Torrance, CA) dissolved in 1 ml acid-saline (pH 2) was given as an intravenous bolus injection. Blood samples for ACTH and cortisol assay were collected at -30, 0, 5, 10, 20, 30, 60, 120 and 180 min through an indwelling i.v. cannula kept open with minute amounts of a diluted (10%) solution of heparin. The cannula was inserted 30 min before CRH administration. Plasma ACTH and cortisol were determined by specific RIAs as described previously (7). The limit of detection of the ACTH assay was 15 pg/ml. To avoid interassay variations all samples of individual patients were determined in the same run.

"High-dose" dexamethasone suppression test

Two mg dexamethasone was given orally q.i.d. for two days. On the second day 24 h urinary excretion of 17-hydroxycorticosteroids (17 OHCS) was measured by gaschromatography (8).

RESULTS

CRH test (Figure 1)

Figure 1 illustrates the individual basal ACTH and cortisol values (i.e. the levels of ACTH and cortisol immediately before CRH injection) as well as

the individual peak ACTH and cortisol levels after CRH administration in the 22 patients with pituitary-dependent Cushing's disease. Twenty of the 22 patients had a clear response of ACTH and cortisol after CRH, although the individual responses were highly variable. Two patients (numbers 21 and 22) with pituitary-dependent Cushing's disease were unresponsive to CRH on two separate occasions.

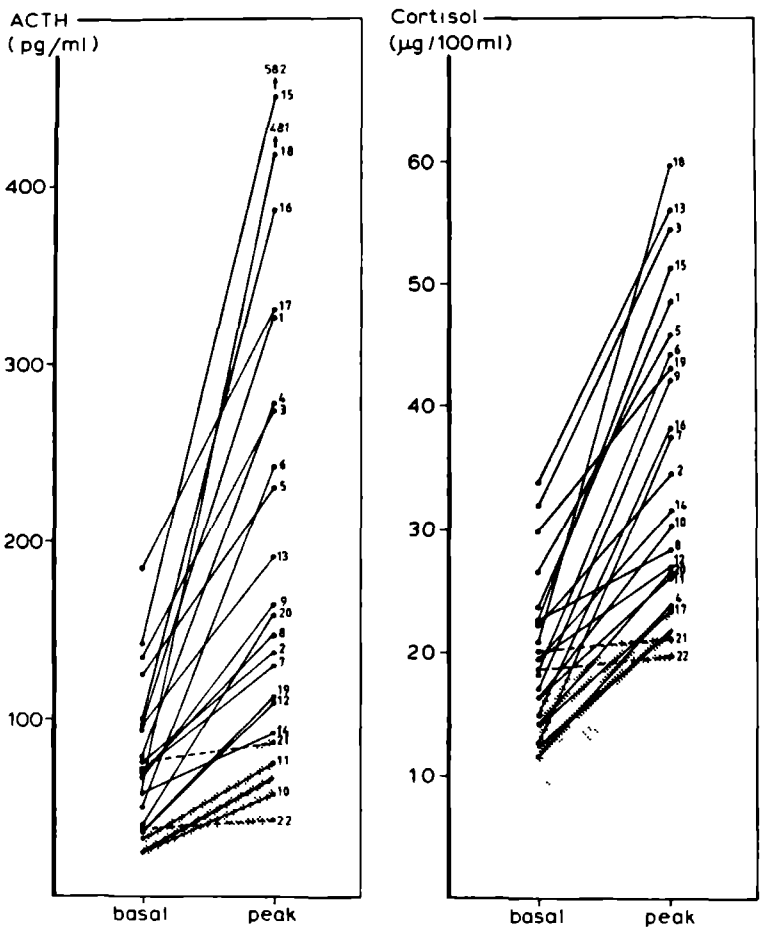

Figure 1. Individual levels of plasma ACTH and cortisol before and after stimulation with 100 μg ovine CRH in 22 patients with pituitary-dependent Cushing's disease Two patients (numbers 21 and 22, dotted lines) were unresponsive to CRH The shaded area represents the mean ± SD in 13 normal subjects (8 men, aged 24 ± 4 years (mean ± SD) and 5 women, aged 31 ± 14 years)

The three patients with Cushing's syndrome due to an adrenal adenoma (numbers 23, 24 and 25, Table 1) were unresponsive to CRH (ACTH levels < 15 pg/ml, both before and after CRH administration). The patient with ectopic ACTH secretion due to a bronchial carcinoid showed no response of ACTH after CRH (basal ACTH level 165.000 pg/ml).

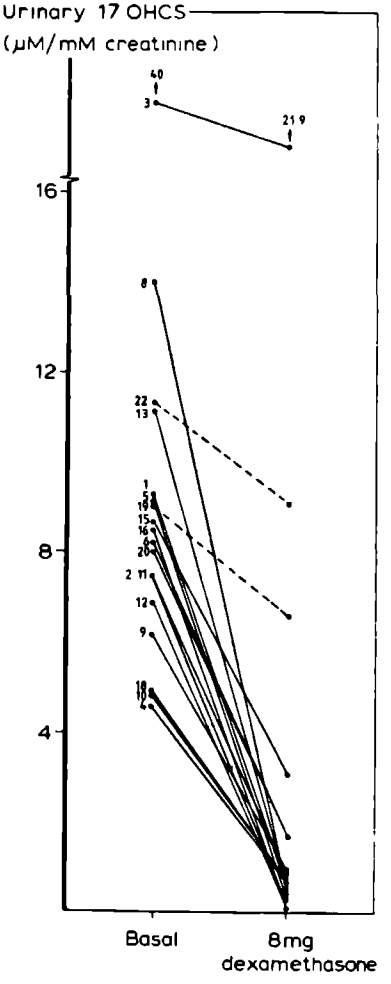

Figure 2 Individual urinary excretion of 17-hydroxycorticosteroids (17 OHCS) before and on the second day of loading with 2 mg dexamethasone orally q i.d in 18 patients with pituitary-dependent Cushing's disease Two patients (numbers 19 and 22, dotted lines) showed inadequate suppression to dexamethasone Normal range for basal urinary 17 OHCS excretion: 0,6 - 3,0 μmol/mmol creatinine (1 μmol/mmol creatinine = 2,48 mg/g creatinine).

"High-dose" dexamethasone suppression test (Figure 2)

Figure 2 illustrates the 24 h urinary excretion of 17-hydroxycorticosteroids before and on the second day of loading with 2 mg dexamethasone orally q.i.d. in 18 patients with pituitary-dependent Cushing's disease. Sixteen of the 18 patients showed adequate (i.e. 40% or more of baseline) suppression of the urinary 17-hydroxysteroid excretion. Two patients (numbers 19 and 22) showed inadequate suppression to dexamethasone. In the remaining four patients with Cushing's disease (numbers 7, 14, 17 and 21, Table 1) data on 17 OHCS excretion was not available; plasma cortisol levels before and after dexamethasone were measured in three of these patients, decreasing from 11,2 to 0,7 μg/100ml after 2 mg dexamethasone q.i.d. for two days in patient 7, from 20,6 to 9,1 μg/100ml in patient 14 and from 21,0 to 8,7 μg/100ml in patient 21.

The three patients with Cushing's syndrome due to an adrenal adenoma failed to suppress after dexamethasone. Urinary 17 OHCS excretion before and after dexamethasone was 4,6 respectively 4,8 μmol/mmol creatinine in patient 23, 4,4 respectively 4,4 μmol/mmol creatinine in patient 24 and 7,2 respectively 6,3 μmol/mmol creatinine in patient 25. Treatment with 2 mg dexamethasone q.i.d. for two days reduced plasma ACTH from 165.000 to 69.000 pg/ml in the patient with ectopic ACTH secretion.

DISCUSSION

The present study compares the value of the CRH test with that of the oral "high-dose" dexamethasone suppression test according to Liddle (5,6) in the differential-diagnosis of Cushing's syndrome. Twenty of the twenty-two patients with pituitary-dependent Cushing's disease responded to CRH with an increase of plasma ACTH and cortisol. From these CRH-responsive Cushing patients one (number 19, Table 1) showed inadequate suppression of urinary 17-hydroxycorticoid excretion in the Liddle test. Two patients with pituitary-dependent Cushing's disease did not respond to CRH. One of these patients (number 22, Table 1) was also unresponsive to dexamethasone. This patient had overt macronodular adrenal hyperplasia. It seems most likely that the unresponsiveness in both tests can be explained in this patient by primacy of adrenal hypercortisolism, as we discussed earlier (9). Very recently a similar patient with Cushing's disease and macronodular adrenal hyperplasia, unresponsive to both CRH and dexamethasone, was described (10). The second patient with unresponsiveness to CRH (number 21, Table 1) also had macronodular hyperplasia, although to a lesser extent. In this patient, unlike in the former, plasma cortisol was reduced considerably (58%) by dexamethasone. Therefore in this case, it is improbable that adrenal primacy can explain unresponsiveness to CRH. In literature up till

now four other patients with Cushing's disease and unresponsiveness to CRH have been described (11-14). It has to be noted that one of these patients (14) did not show suppression of serum cortisol during an 8 mg dexamethasone test either. No results of dexamethasone suppression tests are given for the other three patients.

Our three patients with Cushing's syndrome due to an adrenal adenoma were unresponsive to CRH as well as to dexamethasone. So far no patients with Cushing's syndrome due to an adrenal adenoma or carcinoma responsive to CRH have been reported in literature. Our patient with ectopic ACTH secretion due to a bronchial carcinoid had no plasma ACTH response to CRH, whereas dexamethasone reduced plasma ACTH by 60%. It is of interest, that up till now only one patient with ectopic ACTH secretion responsive to CRH has been reported (15). However, this patient had no response on retesting.

The value of a new diagnostic test has to be proven by comparing it with other diagnostic tests in common use. The oral "high-dose" (8 mg daily for 2 days) dexamethasone suppression test according to Liddle (5,6) is at the present the most reliable test for the differential-diagnosis of Cushing's syndrome. In 1969 Liddle and co-workers (6) reported their results from this test in 100 patients with Cushing's syndrome: 98% of the patients with pituitary-dependent Cushing's disease, 6% of the patients with ectopic ACTH secretion, and none of the patients with adrenal tumors showed adequate suppression of the daily urinary 17-hydroxysteroid excretion, i.e. 40% or more of baseline. Subsequent studies of the 8 mg dexamethasone test have generally shown results comparable to those in Liddle's report. Crapo (16), analysing the cumulative experience in literature of the "high-dose" dexamethasone test, calculated an 8% failure-rate of this test in pituitary-dependent Cushing's disease and a 14% failure-rate in ectopic ACTH syndrome, whereas a false-positive dexamethasone test in patients with adrenal tumors is extremely rare.

Our study demonstrates false-negative responses to CRH in 9% (2 of 22) and to "high-dose" dexamethasone in 11% (2 of 18) of patients with pituitary-dependent Cushing's disease. A false-positive response to dexamethasone occurred in one patient with ectopic ACTH secretion, whereas CRH revealed the cause of Cushing's syndrome in all patients with non pituitary-dependent disease. These results demonstrate that the diagnostic accuracy of the CRH test in the work-up of the patient with Cushing's syndrome is by no means inferior to the Liddle test. It is of interest that in our patients simultaneous failure of both tests to indicate the cause of Cushing's syndrome did not occur, except in the patient with Cushing's disease and severe macronodular hyperplasia. However, taking into account the primacy of adrenal hypercortisolism, the results of both tests in this patient are intelligible.

We recommend to perform routinely a CRH test as well as a "high-dose" dexamethasone test to discriminate with the highest precision between the various forms of Cushing's syndrome. Should conflicting results from both tests and indecisive roentgenological findings occur further diagnostic procedures are necessary, e.g selective venous sampling for ACTH (17).

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3.3 ACTH AND CORTISOL RESPONSES TO OVINE CORTICOTROPHIN-RELEASING FACTOR IN PATIENTS WITH PRIMARY AND SECONDARY ADRENAL FAILURE

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SUMMARY

The ACTH and cortisol responses to an intravenous bolus injection of 100 μg ovine CRF were studied in 19 patients with adrenal failure. In all eight patients with primary adrenal failure, plasma ACTH levels increased from a mean basal level of 1494 ± 431 (SEM) pg/ml to a peak value of 2601 ± 1220 pg/ml at 10 min. In comparison with healthy subjects absolute ACTH increments after ovine CRF were significantly augmented in the patients with Addison's disease ($P^* < 0.001$), and the absolute ACTH responses after ovine CRF were positively correlated with the basal plasma ACTH levels. The 11 patients with secondary adrenal insufficiency could be subdivided into two groups: (A) those having little or no ACTH and cortisol response to ovine CRF (five patients) and (B) those having prolonged and pronounced ACTH responses with a biphasic pattern and a delayed second peak (six patients), followed in all patients by a marked cortisol increase. Clinical and biochemical data in these patients support the view that the CRF test can discriminate between hypothalamic and pituitary causes of secondary adrenal failure.

INTRODUCTION

In the last few years ample studies have demonstrated that ovine corticotrophin-releasing factor stimulates the secretion of ACTH and cortisol in normal human subjects (Grossman et al., 1982; Orth et al., 1983; Hermus et al., 1984) and in the majority of patients with Cushing's disease (Orth et al., 1982; Müller et al., 1983a; Pieters et al., 1983; Chrousos et al., 1984; Lytras et al., 1984).

It has been clearly proven that the response of the pituitary-adrenal axis to ovine CRF in healthy men is modulated to a high degree by the basal cortisol level (Hermus et al., 1984; Lytras et al., 1984), i.e. the higher the basal plasma cortisol level, the smaller the CRF-induced secretion of ACTH and, thereby, of cortisol.

Patients with primary adrenal failure are characterized by a normal hypothalamo-pituitary system, which is not -or only to a minor degree- influenced by endogenous cortisol feedback. Thus in these patients ACTH responsiveness to CRF can be studied independently of circulating cortisol. The present study shows that in these patients the absolute ACTH response to ovine CRF is augmented and positively correlated with the basal ACTH level.

Only scarce data have been published on plasma ACTH and cortisol responses to ovine CRF in patients with secondary adrenal insufficiency. However, there is preliminary evidence that the CRF test will enable us to discriminate between hypothalamic and pituitary disorders in these patients (Tsukada et al., 1984; Schulte et al., 1984). The present study confirms these scarcely reported findings in a substantial group of patients with secondary adrenal failure and shows in addition that the ACTH response to ovine CRF in patients with supposedly hypothalamic adrenal insufficiency is biphasic and more prolonged and pronounced than in normal subjects.

SUBJECTS AND METHODS

Pituitary-adrenal responsiveness to ovine CRF was studied in 19 patients with adrenal failure after approval of the protocol by the hospital ethical committee and obtaining informed consent from all participants. Eight patients had primary and eleven had secondary adrenal insufficiency. The results in the patients were compared with data from ten normal subjects (five men, aged 23 ± 3 years [mean ± SD], and five women, aged 31 ± 14 years). Clinical data of the patients with primary adrenal failure are given in Table 1. The eight Addisonian patients (except for patient 8, who had only partial adrenal insufficiency), were on glucocorticoid and mineralocorticoid replacement therapy at the time of the CRF test. Clinical data of the patients with secondary adrenal insufficiency are summarized in Table 2. At the time of the CRF test patients 9-12, 15-17 and 19 were on glucocorticoid maintainance therapy, whereas patients 13, 14 and 18 had never received glucocorticoid therapy. The CRF test was performed at 0900 h with the subjects fasting and at bed rest. Ovine CRF (100 μg: Bachem, Torrance, CA) dissolved in 1 ml acid-saline (pH 2) was given as an intravenous bolus injection. Blood samples for ACTH and cortisol assay were collected at -30, 0, 5, 10, 20, 30, 60, 120 and 180 min via an indwelling i.v. cannula kept open with minute amounts of a diluted (10%) solution of heparin. The cannula

was inserted 30 min before CRF administration. Plasma ACTH and cortisol were determined by specific RIAs as described previously (Pieters et al., 1982). The limit of detection of the ACTH assay was 15 pg/ml.

To avoid interassay variations all samples of individual patients were determined in the same assay. During the CRF test all patients continued hormonal replacement therapy, but the use of glucocorticoids stopped at least 24 h before each test. Statistical analyses were performed using Wilcoxon's test for paired observations (P-values denoted by P), Wilcoxon's two sample test (P = P*) and Spearman's rank correlation test (P = P**). The mean values ± 1 SEM are given, unless stated otherwise.

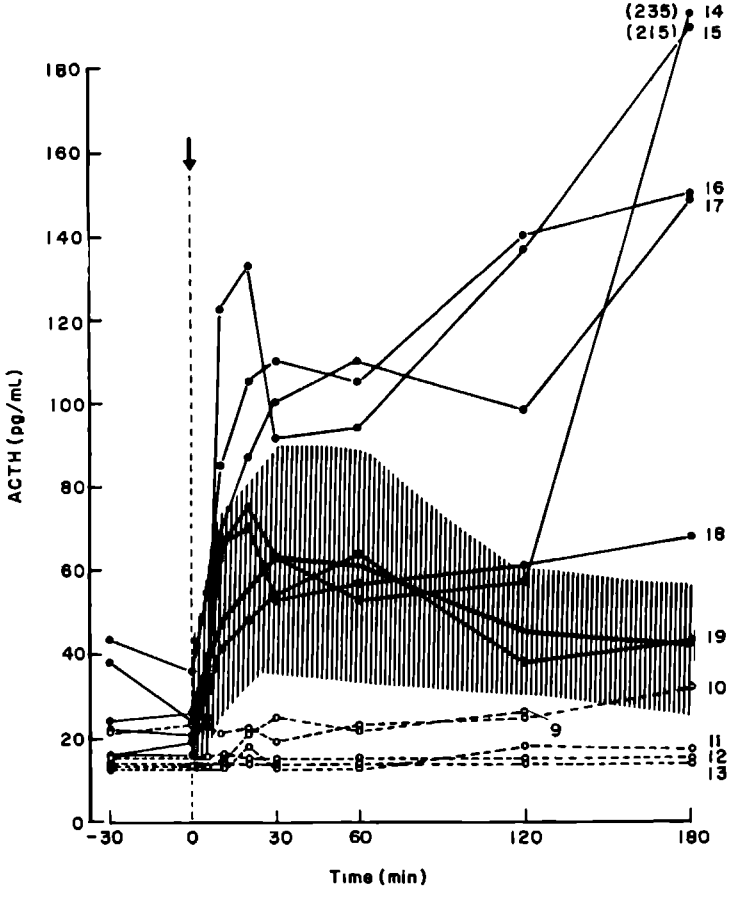

Figure 1 Individual ACTH responses to an intravenous bolus injection of 100 μg ovine CRF (arrow) in 11 patients with secondary adrenal failure. The shaded area represents the mean ± 1 SD in 10 normal subjects

Table 1 Clinical data of the patients with primary adrenal failure

Patient no	Age (years)	Sex	Duration of disease (years)	Etiology	Basal plasma ACTH (pg/ml)	Basal plasma cortisol (μmol/l)
1	26	F	4	Auto-immune	3900	0 02
2	21	F	1	Auto-immune	2500	<0 02
3	30	M	15	Uncertain	1860	<0 02
4	37	F	1	Auto-immune	1950	0 03
5	29	M	10	Uncertain	710	0 12
6	41	M	8	Auto-immune	640	<0 02
7	50	M	<1	Possibly tuberculous	290	0 05
8*	26	F	4	Uncertain	98	0 39

* This patient has only partial adrenal insufficiency Under basal conditions plasma cortisol levels are in the normal range and plasma ACTH levels are only moderately elevated (100-300 pg/ml) There is no plasma cortisol increase after insulin-induced hypoglycaemia or ACTH administration, indicating diminished adrenal reserve Four years ago she had a classical Addisonian crisis, shortly after giving birth to a dead fetus At that time plasma cortisol levels were very low (0.05-0 07 μmol/l)

Table 2 Clinical data of the patients with secondary adrenal failure

Patient no.	Age (years)	Sex	Diagnosis	Duration of disease (years)	Gluco-corticoid treatment	Hyper-prolactin-aemia	Proven deficiencies	Roentgenograms sellar region
9	72	F	Sheehan's syndrome	38	+	-	ACTH, FSH, LH, TSH	Normal plain X-rays
10	42	F	Sheehan's syndrome	2	+	-	ACTH, FSH, LH, TSH, Prol	Normal plain X-rays
11	58	F	Hypopituitarism of unknown origin	24	+	-	ACTH, FSH, LH, TSH, Prol	Normal plain X-rays
12	39	F	Hypopituitarism of unknown origin	14	+	-	ACTH, TSH, Prol, hGH	Normal plain X-rays
13	76	M	Hypopituitarism, probably post-encephalitis	19	-	-	ACTH, FSH, LH, TSH, Prol	Normal plain X-rays, normal CT
14	23	M	Hypopituitarism of unknown origin	14	-	-	ACTH, FSH, LH, TSH, hGH	Empty sella on CT
15	58	F	Hypopituitarism after removal of a large suprasellar craniopharyngioma	4	+	+	ACTH, FSH, LH, TSH	Suprasellar tumour on CT
16	69	F	Hypopituitarism suprasellar tumour	1	+	+	ACTH, FSH, LH	Sellar enlargement on plain X-rays, suprasellar tumour on CT
17	67	M	Hypopituitarism of unknown origin	2	+	-	ACTH, hGH	Normal plain X-rays, normal CT
18	30	M	Hypopituitarism, cerebral haemorrhage post-partum	14	-	+	ACTH, hGH	Normal plain X-rays
19	15	M	Hypopituitarism of unknown origin	10	+	-	ACTH, FSH, LH, TSH, hGH, aVp	Normal plain X-rays

RESULTS

Patients with primary adrenal failure

In all eight patients with proven Addison's disease, ACTH levels increased after ovine CRF injection from a basal value of 1494 ± 431 pg/ml to a peak value of 2601 ± 1220 pg/ml at 10 min. Thereafter ACTH levels declined slowly to 1978 ± 559 pg/ml at 180 min. Individual peak levels were obtained at variable intervals between 10 and 180 min. In none of the patients was the rise in ACTH followed by an increase in cortisol. As compared to the healthy subjects, basal ACTH levels ($P^* < 0.001$), absolute ACTH increments after ovine CRF ($P^* < 0.001$) and peak ACTH levels ($P^* < 0.001$), were significantly augmented in the group of patients with Addison's disease.

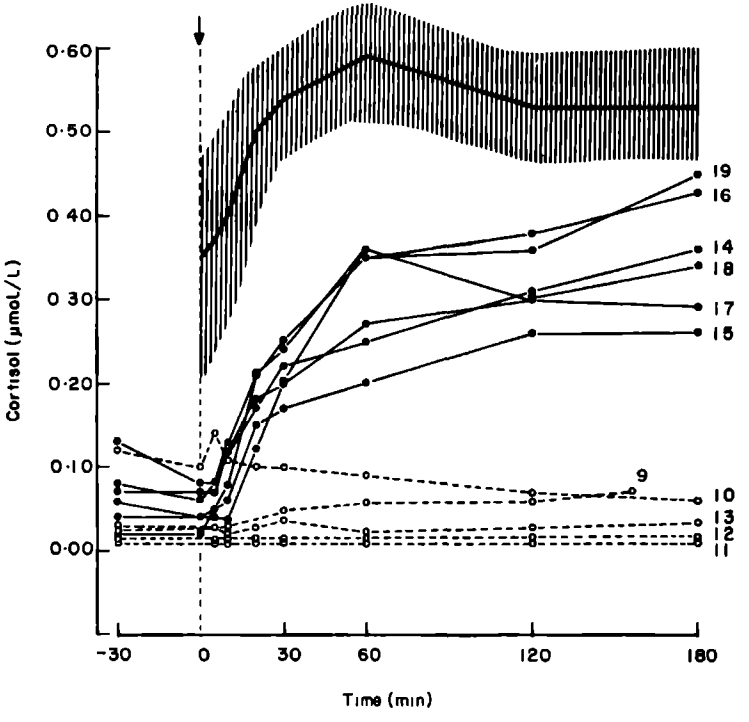

Figure 2. Individual cortisol responses to an intravenous bolus injection of 100 µg ovine CRF (arrow) in 11 patients with secondary adrenal failure. The shaded area represents the mean ± 1 SD in 10 normal subjects.

However, on a percentage basis the increments of ACTH after CRF in Addison's disease (87 ± 20%) were not higher than in normal subjects. In the Addisonian patients no statistically significant correlation, as found in healthy subjects, could be demonstrated between basal plasma cortisol levels and absolute ACTH increments ($r = -0.40$; $P^{**} > 0.10$). However, in the group of Addisonian patients basal plasma ACTH levels correlated positively with the absolute ACTH responses after ovine CRF ($r = +0.81$; $P^{**} < 0.05$).

Patients with secondary adrenal failure

Figure 1 and 2 show the individual ACTH and cortisol responses to oCRF administration in the 11 patients with secondary adrenal failure. Figure 1 clearly illustrates that the 11 patients can be divided into two groups, according to the ACTH responses after CRF administration:

(A) those having little or no ACTH responses to ovine CRF (Patients 9-13) and

(B) those having prolonged and pronounced ACTH responses with (in addition) in five of six cases a biphasic pattern with a delayed second peak (Patients 14-19).

In four of the five patients in group A basal ACTH levels were lower than the limit of detection of the assay. Basal cortisol levels in group A were significantly lower than in the healthy subjects (0.04 ± 0.01 μmol/l vs 0.35 ± 0.04 μmol/l; $P^{*} < 0.01$). In group B plasma ACTH levels rose without exception after injection of the peptide from a basal level of 24 ± 3 pg/ml to 86 ± 14pg/ml at 20 min. Thereafter plasma ACTH levels slightly decreased to 79 ± 12 pg/ml at 30 min and again rose to a second peak of 144 ± 35 pg/ml at 180 min. In all patients of group B plasma ACTH rises were followed by marked increases of cortisol from a baseline value of 0.05 ± 0.01 μmol/l ($P^{*} < 0.01$ vs healthy subjects) to a peak level of 0.36 ± 0.04 μmol/l at 180 min ($P < 0.05$ vs $t = 0$ min). In four of the six patients of group B cortisol levels were still rising at the end of the test. Maximum ACTH increases in group B (124 ± 34 pg/ml) were 2,5 times higher than those in the healthy subjects (49 ± 7 pg/ml), although this difference lacks statistical significance ($0.05 < P^{*} < 0.10$). Maximum cortisol increments in the patients of group B (0.32 ± 0.03 μmol/l) were of a similar order of magnitude to those in the normal controls (0.26 ± 0.03 μmol/l).

DISCUSSION

After the elucidation of the structure of ovine CRF by Vale et al. in 1981, numerous studies have been published on the effects of ovine CRF in patients with Cushing's syndrome (Orth et al., 1982; Müller et al., 1983a;

Pieters et al., 1983; Chrousos et al., 1984; Lytras et al., 1984). However, only scarce and preliminary data have been presented about the use of this peptide as a diagnostic test in patients with adrenal failure (Tsukada et al., 1984; Schulte et al., 1984). In this study, we report upon our experience of the administration of ovine CRF in 19 patients with adrenal failure, either primary or secondary.

In comparison with normal subjects, eight patients with primary adrenal insufficiency demonstrated an elevated basal ACTH concentration and an augmented absolute ACTH response to ovine CRF. This finding confirms preliminary data of Müller et al. (1983b) in two, Nakahara et al. (1983) in two and Schulte et al. (1984) in three patients. Furthermore, we found in our patients with Addison's disease, unlike in healthy subjects, a highly significant and positive correlation between the basal ACTH concentrations and the CRF-induced absolute ACTH increments. We have recently shown that the ACTH and cortisol responses to ovine CRF administration in healthy subjects are highly modulated by the basal cortisol concentrations, i.e. the higher the basal plasma cortisol levels, the lower the CRF-induced secretion of ACTH, and thereby, of cortisol (Hermus et al., 1984). A similar negative correlation between these indices was also demonstrated by Lytras et al. (1984).

Our findings in patients with Addison's disease are the first to demonstrate that, in the absence of cortisol negative feedback, the absolute ACTH response to ovine CRF is augmented and determined by the basal ACTH concentration. It has been reported that adrenalectomy in rats is followed by an increase in hypothalamic immunoreactive CRF (Moldow et al., 1982) whereas there is very preliminary evidence that endogenous CRF levels are high in patients with Addison's disease (Tomori et al., 1983). Combining these data with our own results, it seems that exogenous CRF still stimulates ACTH secretion despite supposedly high endogenous CRF levels.

In patients with secondary adrenal failure, who are known to be characterized by low basal ACTH and cortisol levels unresponsive to insulin-induced hypoglycaemia, our study discloses two subsets of patients according to the ACTH responses after CRF administration: (A) those having little or no ACTH responses to ovine CRF and (B) those having prolonged and pronounced ACTH responses often accompanied by a biphasic pattern with a delayed second peak. It is feasible that the cause of adrenal insufficiency in the latter group is not at the level of the pituitary gland itself, but originates in the hypothalamus or at an even higher level. It appears that the pituitary gland of these patients -although it has probably not been stimulated by endogenous CRF for a long time- is still capable of a prompt release of large amounts of ACTH after CRF injection. The results of this study confirm very recent data of Tsukada et al. (1984) and Schulte et al. (1984), who showed a similar divergence in ACTH responses in

respectively 8 and 6 patients with secondary adrenal failure and reported a similar pattern of ACTH response in patients with supposedly hypothalamic failure. In five out of six of our patients who showed an overt ACTH increase after ovine CRF, a biphasic pattern was observed. This biphasic pattern of ACTH secretion after CRF stimulation has not been reported in healthy subjects tested in the morning hours, but it has been described when the CRF test is performed in the late afternoon (De Bold et al., 1983), at a time when plasma cortisol levels are low. We therefore speculate that the exaggerated and possibly also the biphasic ACTH response to ovine CRF in the patients with presumed hypothalamic failure is uncovered by the lack of cortisol feedback. Our patients with supposedly hypothalamic adrenal failure showed without any exception a cortisol rise of normal magnitude after CRF injection. These data agree with those of Lytras et al. (1984), who demonstrated that six out of seven patients with secondary adrenal insufficiency had normal cortisol responses to CRF. However, only shallow cortisol rises occurred in most of the patients with presumed hypothalamic failure, reported by Tsukada et al. (1984) and Schulte et al. (1984). Our findings demonstrate that the adrenals of some, if not of most patients with secondary adrenal insufficiency maintain their ability to react to CRF-induced ACTH increases for a long time, as has also been demonstrated for exogenous ACTH administration (Cunningham et al., 1983). Obviously our study gives no direct evidence that the patients of group A really have pituitary failure, whereas the patients of group B have hypothalamic or pituitary stalk damage. However, four out of five patients of group A had undetectable prolactin levels, whereas three of the patients of group B had hyperprolactinemia and the others had normal prolactin values. Because prolactin secretion by the pituitary gland is normally under tonic inhibitory control, this marked divergence in prolactin levels provides circumstantial evidenee in favour of the hypothesis that patients of group A have pituitary lesions and patients of group B hypothalamic lesions, as was also suggested by Tsukada et al. (1984). Moreover, two patients of group A had Sheehan's syndrome, suggestive of a pituitary lesion, whereas two patients of group B had suprasellar tumours and one other patient of this group had diabetes insipidus, suggesting hypothalamic or pituitary stalk damage.

Other releasing factors, such as TRH, LHRH and recently GHRH, have been used to discriminate between hypothalamic and pituitary diseases. However, the discriminatory power of single bolus injections of these hypothalamic peptides is poor. Further studies are necessary to demonstrate whether a single bolus injection of CRF can be reliably used to discriminate between primary hypothalamic or pituitary failure. However, it is clear that the CRF test does not make other classical tests, such as the insulin-tolerance test, which documents the integrity of the complete hypothalamo-pituitary-adrenal axis, superfluous in the diagnosis of secondary adrenal insufficiency.

In our opinion, the combination of an insulin-tolerance test and a CRF test will provide optimal information; i.e. insufficient ACTH increase during the insulin-tolerance test will confirm the diagnosis of secondary adrenal insufficiency, whereas CRF administration may potentially differentiate pituitary from hypothalamic causes of this disorder.

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Dr. C.W. Burke (The Radcliffe Infirmary, University of Oxford) commented in a Letter to the Editor in the December 1985 issue of Clinical Endocrinology (p.722):

"Sirs,
Dr. Hermus and his colleagues writing on the ACTH response to CRF in the June issue (pages 761-769) divide their results in ACTH deficient patients into two groups, on the basis of the cortisol response to CRF. They then proceed to speculate that those in group A, patients 9-13, have pituitary failure whereas the patients in group B (numbers 14-19) who show a cortisol response to CRF have hypothalamic failure; but they adduce no evidence whatever to distinguish the two groups pathologically. Perusal of table 1 shows a complete overlap of diagnoses between the two groups.
As evidence of hypothalamic damage the authors adduce the presence of hyperprolactinaemia in two patients, ignoring the fact that hyperprolactinaemia occurs in glucocorticoid insufficiency per se. For example it is seen in Addison's disease (Lever and McKerron, 1984; Refetoff et al 1972) and it is also seen in isolated ACTH deficiency (Burke et al 1979; Yamamoto et al 1976). The presence of hyperprolactinaemia therefore does not distinguish primary from secondary hypoadrenalism, never mind pituitary from hypothalamic ACTH failure."

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We replied (Clinical Endocrinology, 1985, 23: 724):

"Sirs,
Dr. Burke incorrectly states that we divided our patients with secondary adrenal failure on the basis of the cortisol response to CRF. This would not be permissible, because in secondary adrenal insufficiency cortisol responses to ACTH or CRF administration may vary, depending mainly on the severity and the duration of disease. Therefore we divided our patients

Table 1 Clinical and biochemical data of 5 additional patients with secondary adrenal insufficiency

Patient no	Age (years)	Sex	Diagnosis	Duration of disease (years)	Glucocorticoid treatment	Hyperprolactinaemia	Proven deficiencies	Roentgenograms sellar region
20	54	M	Hypopituitarism after removal of a pituitary macroadenoma	1	+	-	ACTH, FSH, LH, TSH, Prol, hGH	No tumour on CT
21	30	F	Hypopituitarism after removal of a large intra- and suprasellar craniopharyngioma	15	+	-	ACTH, FSH, LH, TSH, Prol, hGH	Sellar enlargement on plain X-rays, no tumour on CT
22	22	M	Hypopituitarism, large suprasellar craniopharyngioma	1	-	+	ACTH, FSH, LH, TSH, hGH	Sellar enlargement on plain X-rays, (supra)-sellar tumour on CT
23	33	M	Hypopituitarism, large suprasellar craniopharyngioma	15	-	-	ACTH, FSH, LH, TSH, hGH	Sellar enlargement on plain X-rays, (supra)-sellar tumour on CT
24	54	M	Hypopituitarism after removal of a pituitary macroadenoma with suprasellar extension	1	-	-	ACTH, FSH, LH, TSH, hGH	Sellar enlargement on plain X-rays

Table 2. Basal ACTH and cortisol values and their increases after a bolus injection of 100 μg oCRF in 5 additional patients with secondary adrenal insufficiency

Patient no.	basal ACTH (pg/ml)	Δ max ACTH (pg/ml)	Basal cortisol (μmol/l)	Δ max cortisol (μmol/l)
20	< 15	0	< 0.02	0
21	< 15	0	< 0.02	0
22	< 15	136	0.14	0.49
23	18	165	0.08	0.39
24	< 15	42	0.06	0.53

strictly according to the ACTH response after CRF.

The vital point is to demonstrate that patients with secondary adrenal failure, who do not respond to CRF with an ACTH increase really have a pituitary lesion. Recently we have studied 5 additional patients with secondary adrenal failure (Table 1 and 2) and analysis of the total group of 16 patients provides new evidence of this concept:

I) Six of seven patients with supposedly pituitary failure (group A, patients 9-13 and 20-21) had undetectable prolactin levels, while the patients with supposedly hypothalamic failure (group B) were all normo- or hyperprolactinaemic. This prolactin deficiency in group A patients, even despite the prolactin enhancing effect of glucocorticoid deficiency, is highly suggestive of a pituitary lesion.

II) In group A none of 3 GH deficient patients, none of 4 TSH deficient patients and none of 3 LH/FSH deficient patients responded to a bolus injection of respectively GRF, TRH and LHRH.
In group B the corresponding numbers were 4 out of 5, 5 out of 5 and 4 out of 7 patients.

III) Basal ACTH levels in group A were significantly lower than in group B (16 ± 1 vs 21 ± 2 pg/ml, $P < 0.05$) as were the basal cortisol levels (0.03 ± 0.01 vs 0.07 ± 0.01 μmol/l, $P < 0.05$). This fits well into the concept: we speculate that hypothalamic disease will result in a lesser degree of adrenal failure than primary pituitary disease because intact corticotropic cells, even when deprived of hypothalamic stimulation, will probably secrete small amounts of ACTH."

3.4 COEXISTENCE OF HYPOTHALAMIC AND PITUITARY FAILURE AFTER SUCCESSFUL PITUITARY SURGERY IN CUSHING's DISEASE? [1]

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SUMMARY

The type of secondary adrenal failure, which occurs after successful pituitary surgery in Cushing's disease was studied using CRH administration. Eight patients with Cushing's disease were studied in the immediate postoperative period (9-18 days) of successful pituitary surgery. The results in these patients were compared with those in 13 healthy subjects, 7 patients with secondary adrenal failure of the pituitary type and 9 patients with secondary adrenal failure of the hypothalamic type. In all postoperative Cushing patients, except one, clear ACTH and cortisol responses occurred after CRH, demonstrating hypothalamic adrenal failure in these patients. This demonstration of hypothalamic adrenal failure after neurosurgery strongly argues against a pivotal role for endogenous CRH in the pathogenesis of Cushing's disease in these patients. The mean ACTH response to CRH in the postoperative Cushing patients was significantly lower than that in patients with adrenal failure of the hypothalamic type. This suggests the presence of pituitary failure in post-adenomectomy Cushing patients.
In conclusion, this study provides arguments that the secondary adrenal failure after adenomectomy in Cushing's disease is caused by both hypothalamic and pituitary failure.

INTRODUCTION

Successful treatment of Cushing's disease by pituitary surgery leads to a transient period of postoperative adrenal failure (1-4). It has been demon-

[1] Presented in part at the Second Meeting of the European Neuroendocrine Association (Abstract 14), Milan, October 15-17, 1985.

Submitted for publication

strated that the adrenal failure in these patients is of the secondary type (1-4). It is not yet known whether this secondary adrenal failure is caused by hypothalamic or pituitary suppression, or both. Demonstration of hypothalamic dysfunction after treatment for Cushing's disease has etiological implications, because this would strongly argue against a central role for endogenous CRH in the pathogenesis of Cushing's disease.

Recently others (5,6) and we (7,8) adduced evidence that the CRH test can discriminate between hypothalamic and pituitary causes of secondary adrenal insufficiency. The present study was designed to characterize the type of secondary adrenal failure in patients with Cushing's disease, shortly after successful pituitary surgery by using CRH administration.

PATIENTS AND METHODS

Pituitary-adrenal responsiveness to ovine CRH was studied in eight patients with Cushing's disease in the preoperative and immediate postoperative period (9-18 days) of successful pituitary surgery. Clinical and biochemical data of these patients are given in Table 1. All female patients were premenopausal. Preoperatively all 8 patients had clear (hyper-) responses of ACTH and cortisol after CRH administration. Patients 1 and 7 were operated via a transfrontal approach, the other six patients via the transsphenoidal route. On the day of surgery and during the first two postoperative days the patients received 75 mg prednisolone intravenously. Thereafter glucocorticoid therapy was tapered off and stopped seven days after the operation, i.e. at least 48 hrs before the postoperative CRH test. Postoperative endocrine evaluation revealed hypocortisolism in all cases, necessitating glucocorticoid replacement therapy which was started after the CRH test. Except in patient 1, who developed secondary hypothyroidism and hypogonadism after operation, no further replacement therapy was necessary.

The results in these patients were compared with data from 13 healthy subjects (8 men, aged 24 ± 4 years (mean $\pm$ SD) and 5 women, aged 31 ± 14 years) and with that of 16 patients with secondary adrenal failure, due to various causes. The clinical data and the results of the CRH tests in these patients have been published previously (7,8). According to the ACTH responses after CRH administration, these 16 patients were subdivided into those supposed to have pituitary (n=7) or hypothalamic (n=9) failure.

The CRH test was performed at 9 a.m. with the subjects fasting and at bed rest. Ovine CRH (100 μg: Bachem, Torrance, CA) dissolved in 1 ml acid-saline (pH 2) was given as an intravenous bolus injection. Blood samples for ACTH and cortisol assay were collected at -30, 0, 5, 10, 20, 30, 60, 120 and 180 min via an indwelling i.v. cannula kept open with minute amounts of a diluted (10%) solution of heparin. The cannula was inserted 30 min before

Table 1 Clinical and biochemical data of eight patients with pituitary-dependent Cushing's disease

Patient no	Age (years)	Sex	Duration of disease (years)	Pre-operatively			Post-operatively		
				Basal ACTH[a] (pg/ml)	Basal cortisol[b] (μg/dl)	Cortisol after 2 mg DXM q.i.d. for 2 days (μg/dl)	Postoperative CRH test on day	Length of follow-up (months)	Glucocorticoid replacement (months)
1	53	M	1	85	27,2	2,5	15	17	≥17
2	20	F	2	51	21,0	0,7	10	died on day 25 p.o. (pulmonary embolism)	
3	35	F	0,5	140	24,3	8,7	15	41	≥41
4	32	F	1	62	21,7	9,1	18	15	11
5	40	M	1	35	18,5	2,2	9	12	≥12
6	38	M	5	49	15,2	1,1	17	9	≥ 9
7	24	F	2	39	21,0	7,2	13	9	≥ 9
8	34	F	3	53	27,5	6,9	10	35	17

a Basal ACTH represents the mean of 4 samples taken at 9 a.m. (normal value < 75 pg/ml).

b Basal cortisol represents the mean of 4 samples taken at 9 a.m. (normal range 6,9 - 19,9 μg/dl).

CRH administration. Plasma ACTH and cortisol were determined by specific RIAs as described previously (9). The limit of detection of the ACTH assay was 15 pg/ml. To avoid interassay variations all samples from the individual patients were determined in the same run. Statistical analyses were performed using Wilcoxon's two sample test. The mean values ± 1 SEM are given, unless stated otherwise.

RESULTS (Figure 1-3, Table 2)

Healthy subjects and patients with secondary adrenal failure of the pituitary or hypothalamic type

Table 2 gives the basal values for ACTH and cortisol and the peak values and maximal increments after CRH administration for these 3 groups. Maximal ACTH increases after CRH were significantly higher ($P < 0,05$) in the patients with secondary adrenal failure of the hypothalamic type than those in the healthy subjects, as were the maximal cortisol increases ($P < 0,05$). However, despite ACTH hyperresponsiveness peak cortisol levels after CRH were significantly lower ($P < 0,05$) in these patients than in the healthy subjects.

Patients with Cushing's disease after successful pituitary surgery

Figure 1 illustrates the individual ACTH and cortisol responses to 100 μg ovine CRH in the immediate postoperative period in the eight patients with pituitary-dependent Cushing's disease. In six of the seven patients, in whom ACTH levels were determined, basal ACTH was close to (in one patient) or lower than (in five patients) the limit of detection of the ACTH assay. In all eight patients basal cortisol was less than 3,3 μg/dl, except in one patient who had a basal cortisol level of 7,2 μg/dl. Figure 1 shows that in all Cushing patients after surgery CRH administration caused pituitary-adrenal activation, except in patient 3. This patient could not be weaned from glucocorticoid replacement therapy as yet (41 months after surgery).

In Table 2 and Figure 2 the basal values for ACTH and cortisol and their responses after CRH administration in the eight patients with Cushing's disease after operation are compared with the corresponding values before operation. In spite of a much higher baseline cortisol level preoperatively, the ACTH response to CRH was more pronounced before surgery in comparison with after surgery in all patients. There was no significant correlation between the response of the pituitary-adrenal axis to CRH in the individual patients on both occasions.

Figure 3 illustrates the mean ACTH and cortisol responses to CRH in the eight Cushing patients post-adenomectomy and compares these responses

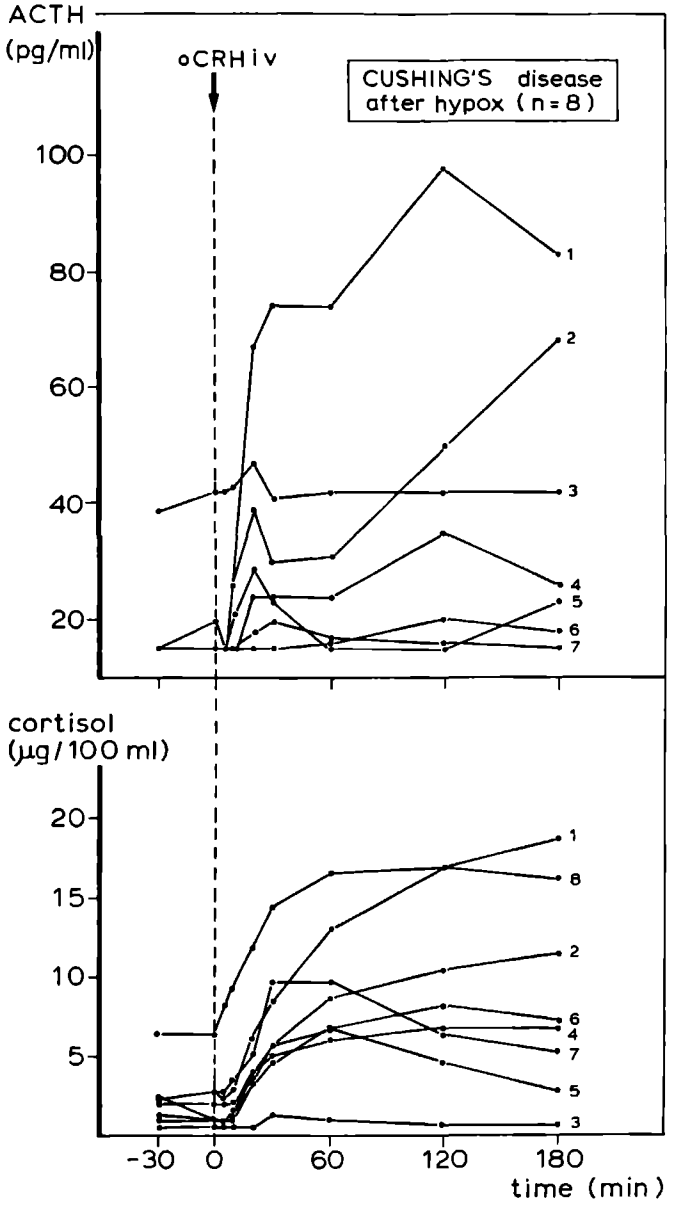

Figure 1 Individual ACTH and cortisol responses to an intravenous bolus injection of 100 µg ovine CRH in 8 patients with pituitary-dependent Cushing's disease in the immediate postoperative period (9-18 days) of successful pituitary surgery (hypox. = pituitary surgery).

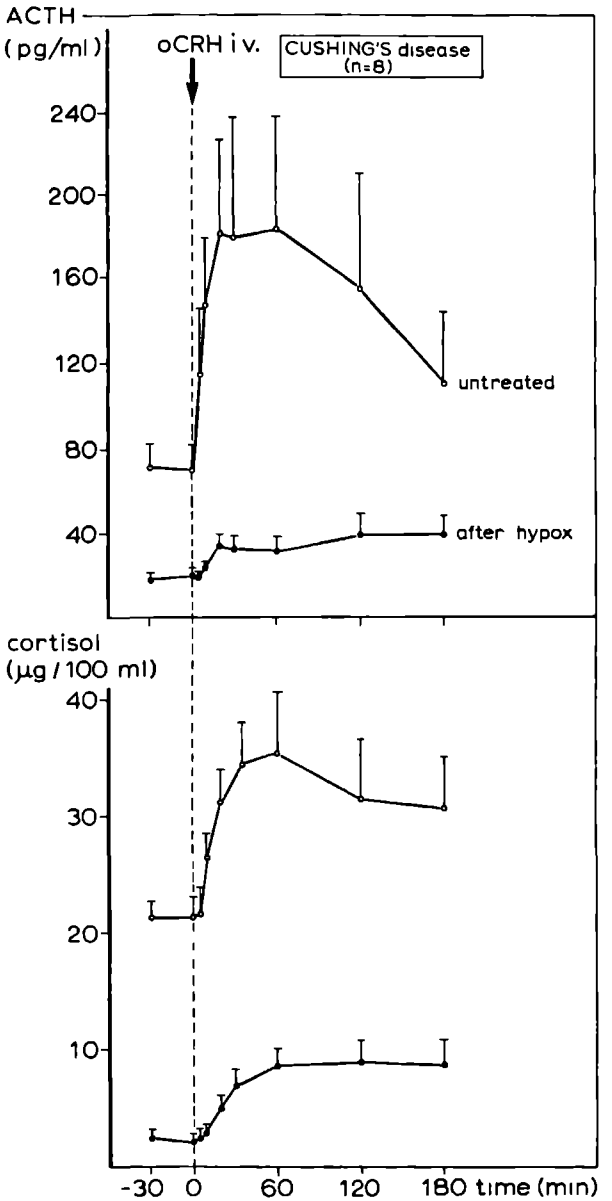

Figure 2. Mean ± SEM plasma ACTH and cortisol levels after an i.v. bolus injection of 100 µg ovine CRH in 8 patients with pituitary-dependent Cushing's disease before and immediately (9-18 days) after successful pituitary surgery (= hypox).

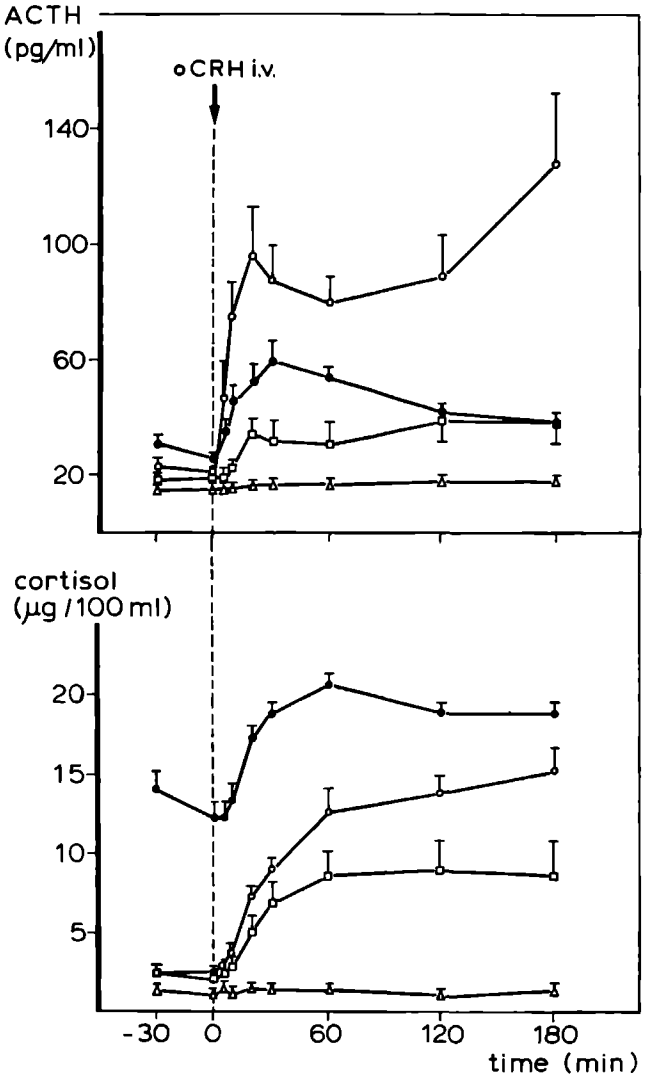

Figure 3. Mean ± SEM plasma ACTH and cortisol levels after an i.v. bolus injection of 100 µg ovine CRH.

●——● normal subjects (n=13)

△——△ patients with secondary adrenal failure, pituitary type (n=7)

o——o patients with secondary adrenal failure, hypothalamic type (n=9)

□——□ patients with pituitary-dependent Cushing's disease, shortly after successful pituitary surgery (n=8)

with those of the healthy subjects and the patients with secondary adrenal failure of the pituitary or hypothalamic type. The maximal ACTH and cortisol responses after CRH in the Cushing patients after surgery were significantly higher than those in the patients with secondary adrenal failure of the pituitary type, but significantly lower than those in the patients with secondary adrenal failure of the hypothalamic type (Table 2).

DISCUSSION

It is still unknown whether the secondary adrenal failure associated with successful selective removal of ACTH secreting pituitary adenomas is caused by insufficient hypothalamic or pituitary drive of adrenocortical function, or by a combination of both. A number of studies have demonstrated that CRH administration discloses two subsets of patients with secondary adrenal failure: (i) those who have little or no ACTH responses to CRH and (ii) those who have prolonged and pronounced ACTH responses to CRH (5-8). A number of clinical and biochemical data support the view that the secondary adrenal failure in the first group of patients is caused by a pituitary lesion and in the second group by a hypothalamic lesion (5-8). It has to be noted that the final proof for this concept is still lacking. Nevertheless, a conclusion that can be safely drawn is that patients with secondary adrenal insufficiency, who respond to exogenous CRH, have at least hypothalamic failure.

The present study illustrates that in the immediate postoperative period all eight patients cured of Cushing's disease were hypocortisolemic, whereas CRH administration caused pituitary-adrenal activation in all but one. Müller et al (10) and Chrousos et al (11) also demonstrated responsiveness of the pituitary-adrenal axis to CRH shortly after successful neurosurgery in one and three patients respectively. As is reasoned above, this pattern strongly suggests insufficient hypothalamic drive in these Cushing patients immediately after pituitary surgery. This observation strongly argues against a pivotal role for endogenous CRH in the pathogenesis of Cushing's disease in these patients. Recent observations of low CRH levels in cerebrospinal fluid (12) as well as in peripheral plasma (13) in pituitary-dependent Cushing's disease are in line with this conclusion.

This study also shows that the mean maximal ACTH response after CRH in the Cushing patients after surgery was significantly lower than that in an earlier published group of patients with secondary adrenal failure of the hypothalamic type (7,8). This suggests that at the time of testing the pituitary reserve in the postsurgery Cushing patients is subnormal, either due to the surgical procedure per se or - and that seems more likely - as a result of the suppressive action of preoperative hypercortisolism on the non-tumorous corticotrophs, which frequently show Crooke's changes (1) and contain less

Table 2. Plasma ACTH and cortisol: Basal values, peak values after 100 μg oCRH and maximal increments after 100 μg oCRH in 13 healthy subjects, 7 patients with secondary adrenal failure of the pituitary type, 9 patients with secondary adrenal failure of the hypothalamic type and 8 patients with Cushing's disease (CD) before and after successful pituitary surgery

		Plasma ACTH (pg/ml)[a]			Plasma cortisol (μg/dl)[a]		
		Basal value	Peak value	ΔMax	Basal value	Peak value	ΔMax
I	Healthy subjects	25 ± 2	67 ± 6	42 ± 6	12,3 ± 1,1	21,7 ± 0,7	9,4 ± 1,1
II	Sec.adrenal failure, pituitary type	16 ± 1	19 ± 2	3 ± 2	1,1 ± 0,4	1,8 ± 0,7	0,4 ± 0,4
III	Sec.adrenal failure, hypothalamic type	21 ± 2	139 ± 22	118 ± 22[b]	2,5 ± 0,4	15,6 ± 1,4[b]	13,4 ± 1,1[b]
IV	CD before surgery	70 ± 12	249 ± 61	179 ± 53	21,4 ± 1,8	42,0 ± 4,3	20,6 ± 4,0
V	CD after successful surgery	20 ± 3	45 ± 10	26 ± 11[c,d]	2,2 ± 0,7	10,1 ± 1,8	8,0 ± 0,4[c,d]

a mean ± SEM b $P < 0,05$ vs I c $P < 0,05$ vs II d $P < 0,05$ vs III

than normal ACTH (14) in Cushing's disease. It cannot be excluded, but it seems unlikely, that the postoperative treatment with glucocorticoids during 7 days has influenced the test results in these patients, who had long-standing hypercortisolism preoperatively. Insufficient ACTH responsiveness to CRH in the immediate postoperative period after neurosurgery for Cushing's disease has also been suggested by Orth et al (15) in two patients and by Hotta et al (16) in five patients.

In summary, our study provides arguments for the conclusion that the secondary adrenal failure after successful pituitary surgery for Cushing's disease is caused by both hypothalamic and pituitary suppression. This coexistence of hypothalamic and pituitary failure postoperatively fits well in the concept that long-term hypercortisolism suppresses the hypothalamic-pituitary-adrenal axis at both the hypothalamic and pituitary level.

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Chapter 4

Safety of corticotropin-releasing hormone

In this thesis our experience with the intravenous administration of CRH in man is reported. Between May 1982 and December 1985 we performed 330 "CRH tests". 25% of the tests were done in healthy volunteers and 75% in patients with a variety of endocrine disorders. In 20% of the tests human CRH was used and in 80% ovine CRH. CRH was obtained from Bachem Inc., Torrance, CA, U.S.A. As described in Appendix 1, the product was prepared for human use by the Department of Clinical Pharmacy, Sint Radboud Hospital (Drs.J.H.Bakker).
The CRH tests were carried out as described in the methods sections of the previous chapters. During each test blood pressure, pulse rate and electrocardiogram (Roche "Arteriosonde" 1225, monitor 103) were monitored.

In the vast majority of patients the test procedure was much better tolerated than more classical stimulation tests of the pituitary-adrenal axis, e.g. the insulin-hypoglycemia test, the lysine-vasopressin test and the pyrogen test. In most subjects facial flushing occurred after the administration of 100-200 μg CRH, in some subjects together with mild dyspnea (subjective sensation of shortness of breath, accompanied by the objective finding of deep and more rapid respirations, lasting 30 sec or less). In seven tests we observed an adverse reaction.

ADVERSE REACTIONS

During the first year of this study we used a relatively high dose of 200 μg ovine CRH. In that period we observed adverse reactions in four patients. In two patients with pituitary-dependent Cushing's disease and in one with secondary adrenal insufficiency an "absence-like" loss of consciousness was seen which started within a few seconds of the injection of CRH and lasted for from 10 sec to 5 min. In one patient with secondary adrenal failure (case A, Appendix 2) a profound fall in blood pressure, followed by asystole occurred after CRH administration.

We reported these adverse reactions in a Letter in The Lancet (1) and sent a vial with ovine CRH from the batch, which had given the adverse reaction in patient A, to Dr. P.J. Lowry (Protein Hormone Unit, St. Bartholomew's Hospital, London) for analysis. No impurities were found in the preparation. Schulte et al (National Institutes of Health, Bethesda, USA) (2) in a reaction to our report suggested that the side-effects, described by us, were due to the relatively high dose of CRH. From February 1983 onwards we lowered the dose of CRH to 100 μg. Thereafter we never again observed a disturbance of consciousness after CRH injection. Our finding that 100 μg ovine CRH does not cause a lowering of diastolic blood pressure in healthy subjects, whereas 200 μg ovine CRH decreases diastolic blood pressure by 14 mm Hg on the average (Chapter 2.3) strengthened our belief of a dose-dependency of the observed side-effects.

During the next three years hypotension during a CRH test occurred in three cases (patients B to D, Appendix 2). However, the delay of 2½ hours between the injection of CRH and the fall in blood pressure in patient B, respectively the beginning of the symptoms before the injection of CRH in patient C, makes it unlikely that in these cases the fall in blood pressure is caused by CRH itself. In both patients a vaso-vagal reaction is more likely, although we cannot rule out that the symptoms were aggravated by CRH. A third hypotensive episode with bradycardia occurred in May 1985 in a 25 year old woman with secondary adrenal failure after successful neurosurgery for pituitary-dependent Cushing's disease three months earlier (case D, Appendix 2). It has to be stressed that in this woman the fall in blood pressure occurred after infusion of as less as 55 μg ovine CRH, whereas bolus injection with 100 μg ovine or human CRH in this patient at three previous occasions had caused no cardiovascular adverse effects at all.

Meanwhile we had received the answers to a questionnaire, concerning side-effects of CRH administration in man, which we had sent to 9 research centers with major experience with CRH in humans. The names of these centers as well as some details about the CRH preparations and their use are given in Table 1. One case of hypotension after CRH in a healthy subject, who fainted after venepuncture and before receiving CRH and whose blood pressure dropped further at 5 min after CRH from 90 to 60 mmHg systolic was reported by Espiner (Cfr. our case C). None of the other investigators reported hemodynamic problems after CRH administration.

We wondered again whether the CRH preparation used by us contained some impurities and once more we decided to send samples from our human CRH preparation (from the batch, used in patient C) and from our ovine CRH preparation (from the batch, used in patient D) to an independent investigator (Dr. G.P. Chrousos, National Institutes of Health, Bethesda U.S.A.) for analysis. Both the human and ovine CRH vials were found to be sterile and non-pyrogenic by the Bureau of Biologics, FDA. HPLC analysis of our product compared to the NIH product showed similar retention times and peak heights (i.e. similar identity and quantity) and no evidence of impurities.

Then we wondered whether the hypotensive reaction in patient D could be caused by an interaction between CRH and the heparin solution (see Appendix 1), still present in the intravenous cannula after flushing. A strong argument for an interaction between both molecules provided the observation by the NIH workers that the addition of 500 IU/ml heparin to ovine CRH resulted in a complete loss of the CRH peak on HPLC. Further studies (in collaboration with Professor C.W. Hilbers, Department of Biophysical Chemistry, University of Nijmegen) to prove and characterize this possible interaction between CRH and heparin are in progress. We think it is not unlikely that the hypotension in patient D is caused by an interaction

Table 1. Research centres participating in the questionnaire about the side-effects of CRH. Some details on the CRH preparations and their use in these centers.

Author	CRH synthesized by	Dose	Dissolved in
G.P. Chrousos, NIH Bethesda, U.S.A.	Bachem Inc.	1 μg/kg	Sterile water with 5% mannitol
D.N. Orth, Nashville, U.S.A.	Dr. J. Rivier (Salk)	1 μg/kg	Saline, containing 10% mannitol, 0,25% human serum albumine and 0,9% benzyl alcohol.
G M. Besser, London, U.K.	Dr. D. Coy (New Orleans)	100 μg	Acid-saline (pH 2)
G. Copinschi, Brussels, Belgium	U.C.B. Bioproducts, Brussels	50 μg	Acid-saline (pH 2)
O.A. Müller, Munchen, F.R.G.	Bachem Inc.	100 μg	Acid-saline (pH 2)
H.M. Schulte, Essen, F.R.G.	Bachem Inc.	1 μg/kg	Sterile water with 5% mannitol
E.A. Espiner, Christchurch, New Zealand	Bachem Inc.	200 μg	Acid-saline
H. Imura, Kyoto, Japan	Peptide Institute, Osaka	100 μg	0,9% NaCl
M. Nakahara, Tokyo, Japan	Dr. N. Ling (Salk)	100 μg	0,9% NaCl
This study	Bachem Inc.	100-200 μg	Acid-saline (pH 2)

between CRH and heparin, comparable with the well-defined potentiating effect of heparin on protamine-induced hypotension (3). However, it has to be noted that in preliminary experiments in anaesthetized dogs and in conscious spontaneously hypertensive rats (in collaboration with Dr. J.F M. Smits, Department of Pharmacology, University of Maastricht) we were not able to prove a potentiation of the hemodynamic effects of CRH injection by mixing CRH with heparin (data not yet reported).

Preliminary conclusions

1. The experience with CRH in a number of qualified centres all over the world demonstrates that ovine CRH in doses of 100 μg or less is a safe agent for testing the pituitary-adrenal axis in man.
2 The major adverse effects observed in a small minority of patients after CRH injection in our center are not caused by an impurity of the preparation The side-effects occurring in four patients during the first year of this study were most likely caused by a too high dose of the peptide The only major hypotensive reaction, unequivocally related with the lower dose of CRH may be caused by a not yet delineated interaction between CRH and heparin Therefore we recommend to omit the use of heparin solutions to keep open i v. cannulas in CRH test procedures.

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APPENDIX 1

Preparation of CRH by the Department of Clinical Pharmacy, Sint Radboud Hospital, University of Nijmegen, The Netherlands

CRH, either ovine or human, obtained in lyophilized form from Bachem Inc., Torrance, CA, USA, is dissolved in a 2% aqueous solution of lactose and sterilized by passage through a 0,2 μm cellulose acetate filter (Schleicher and Schuell, FPO 30/3) Containers with 1 ml of solution are freezedried. The lyophilized product is stored at -18°C in sterile vials under vacuum (1

vial containing 100 μg CRH and 20 mg lactose). Immediately before administration the lyophilized material is dissolved in 1 ml acidified saline (HCl-NaCl, pH 2)/100 μg CRH. Pyrogen tests ("Pyrogent test", Mallinckrodt) are regularly carried out

Preparation of a 10% solution of heparin, used for prevention of clotting in the i.v. line

One ml of heparinum natricum (Tromboliquine, Organon Teknika, Boxtel, Holland; 1 ml = 5000 I.U. heparin) is diluted in 9 ml NaCl 0,9% to a final solution of 500 I.U. heparin/ml.

APPENDIX 2

Case reports

Patient A is a 58 year old woman on cortisone and triiodothyronine replacement therapy after removal of a large craniopharyngioma four years earlier, who had a Spitz-Holter drain for treatment of internal hydrocephalus.
On February 15th, 1983, she received 200 μg ovine CRH as a bolus injection after withdrawal of cortisone for 24 hours. Within 3 minutes of the injection of CRH her systolic blood pressure dropped from 109 to 64 mmHg, followed by a decrease in pulse rate from 81 to 39 /min after 7 minutes, at which time an ECG revealed an AV-junctional rhythm. Immediately afterwards asystole ensued, which could immediately be reversed by a precordial blow. Thereafter the heart regained a sinus rhythm of 72/min and the blood pressure returned to normal. This patient had no history of signs or symptoms of heart disease and an ECG was normal before and after CRH testing.

Patient B is a 72 year old woman, known since 1970 with hypopituitarism, on replacement therapy with cortisone and thyroxine. On April 4th, 1984, she received a bolus of 100 μg ovine CRH after withdrawal of cortisone for 24 hours. During the first hours after CRH injection she had a stable blood pressure (160/90 mmHg). Two and a half hour after the administration of CRH she complained of dizziness, despite being in recumbent position. At that time the pulse rate was 50/min and blood pressure was immeasurably low. After injection of 1/4 mg atropine and infusion of 250 ml plasma blood pressure and pulse rate normalized within 5 minutes. An ECG and results of blood chemistry excluded the possibility of myocardial injury.

Patient C is a 24 year old healthy, well-trained, male medical student. A complete physical examination, routine biochemistry and an ECG were completely normal. On March 27th, 1985, he was tested with human CRH. An i.v. forearm cannula was inserted one hour before CRH injection. Blood pressure and pulse rate before the injection of the peptide were 108/77 mmHg respectively 52/min. At the moment of injection he said he feeled unhappy and dizzy, which started 5 seconds *before* the injection. The injection was immediately terminated and he received only 50 μg in stead of 100 μg human CRH. Immediately afterwards the ECG revealed an AV-junctional rhythm of 33/min and the blood pressure was immeasurably low. The patient was laid down in Trendelenburg position, 1/2 mg atropine was injected and plasma infusion was started. Within 5 min after the start of the symptoms blood pressure increased to 80/60 mmHg and 10 min later after injection of another 1/4 mg atropine, sinus rhythm restored. An ECG revealed no abnormalities any longer. It has to be stressed that the patient never lost consciousness during this period.

Patient D is a 25 year old woman with pituitary-dependent Cushing's disease. On August 29th, 1984, she was tested with a bolus injection of 100 μg ovine CRH. On September 7th, 1984, she received 100 μg human CRH. Shortly after successful pituitary surgery on February 7th, 1985, she was retested with 100 μg ovine CRH. No cardiovascular adverse effects occurred during the first three CRH tests. Because of postoperative hypocortisolism cortisone acetate was started. On May 7th, 1985, 36 hours after the last cortisone dose, a continuous i.v. infusion was given with ovine CRH (100 μg ovine CRH in 15 min). Two i.v. lines were inserted 1/2 hour before starting the infusion. Blood pressure was 120/80 mmHg before CRH. Four minutes after starting the CRH infusion the i.v. line was flushed with $\pm$ 3 ml of a 10% solution of heparin (1 ml = 500 I.U. heparin) because of clotting. Within a few minutes after restarting the CRH infusion blood pressure dropped to 88/53 mmHg, immediately followed by disappearance of sinus rhythm and appearance of an AV-junctional rhythm of 47/min. CRH infusion was stopped (at that time 55 μg ovine CRH was infused) and 1/2 mg atropine was given i.v. Blood pressure and heart rate recovered within a few minutes. However periods with an AV-junctional rhythm were seen till 18 min after the start of the CRH infusion.

Chapter 5

Comments

Physiology and pathophysiology

The most striking observation of this thesis, described in Chapter 2.1, is that the lower the basal plasma cortisol concentration in healthy subjects, the higher the integrated plasma ACTH response to ovine CRH. Remarkably, no significant correlation, either positive or negative, was found between the basal plasma ACTH level and the response of this hormone to ovine CRH. This study was the first to demonstrate the modulatory effect of physiological cortisol levels on the ACTH response to CRH in man. These observations suggest that the response of the pituitary to CRH is primarily dictated by an extrinsic factor, i.e., by the level of circulating glucocorticoids, rather than by the pituitary gland itself. A pharmacological argument for this concept was found in the study reported in Chapter 2.4, in which we demonstrated a significant inverse correlation between the plasma ACTH and cortisol responses to ovine CRH in healthy subjects after overnight suppression with increasing doses of dexamethasone and the plasma level of the synthetic glucocorticoid immediately before injection of CRH. In order to prove unequivocally that physiological concentrations of cortisol inhibit the response of the hypophysis to CRH we investigated whether after pretreatment with the antiglucocorticoid RU-486 the CRH induced ACTH rise is enhanced (Chapter 2.5). First, we found that a single dose of 100 mg of the antiglucocorticoid at 02.00 h leads to a significant increase of the mean ACTH and cortisol level six to seven hours later. The most ready explanation for this concurrence of elevated ACTH and cortisol levels after blocking glucocorticoid activity is that the antiglucocorticoid, by occupying glucocorticoid receptors at the level of the pituitary and/or hypothalamus, attenuates the inhibitory effect of circulating cortisol leading to elevated ACTH levels, which in turn leads to elevated cortisol levels. In line with this effect we found that, despite a higher mean basal cortisol level, loading with this competitive antagonist of cortisol significantly increases the mean maximal ACTH rise to human CRH. In our opinion this observation is the strongest argument so far obtained for the concept that, in man, physiological concentrations of cortisol determine the response of the pituitary to CRH.

After these observations in healthy subjects we analysed which factor determined the ACTH response to CRH in patients with Addison's disease - a condition characterized by absence of cortisol feedback - and in patients with pituitary-dependent Cushing's disease - a condition characterized by a deficient cortisol feedback. We found that in both disorders the absolute ACTH response to ovine CRH is augmented and positively correlated with the basal ACTH level, whereas no correlation could be demonstrated between the basal plasma cortisol level and the response of the pituitary to CRH (Chapters 3.1 and 3.3). As far as Addison's disease is concerned, we

speculate that the virtual absence of circulating cortisol in this disorder reveals the primacy of the basal activity of the corticotrophs in their responsivity to CRH. In Cushing's disease, however, we hypothesize that the primacy of the basal activity of the corticotrophs in the response to CRH emerges as a consequence of the primary defect in the receptiveness of the corticotrophs for circulating cortisol.

At the end of 1983 *human* CRH became available for clinical investigations. In Chapter 2.2 we demonstrated that human CRH has a much shorter duration of action than ovine CRH in man. This has been confirmed by Schürmeyer et al (1). These workers also demonstrated that the stimulation of the hypophyseal-adrenal axis by human CRH is shorter than after ovine CRH injection and that this difference is caused by an approximately 3 to 4 times higher metabolic clearance rate of the former peptide. In Chapter 2.3 we showed that in healthy subjects bolus injections of 200 μg of both ovine and human CRH caused a significant decrease in diastolic blood pressure, which was not observed after the 100 μg doses of both peptides. Moreover, we showed that human CRH caused more pronounced hypotension than ovine CRH in man, and therefore we prefer ovine CRH in everyday practice.

Clinical considerations

In Chapter 3.3 we adduced evidence that a single bolus injection of CRH could divide patients with secondary adrenal failure into those with hypothalamic and those with pituitary disease. We commented that the secondary adrenal failure, which occurs after successful pituitary surgery in pituitary-dependent Cushing's disease is caused by coexistent pituitary and hypothalamic failure (Chapter 3.4).

Recently we studied the type of the transient adrenal failure after unilateral adrenalectomy in two patients with Cushing's syndrome due to an adrenal adenoma. The results of the CRH tests in these patients, before and after operation, are illustrated in Figure 1. It shows that in both patients the adrenal failure immediately after operation is of the combined hypothalamic-pituitary and adrenal type, and that the recovery of the hypothalamic-pituitary function precedes the recovery of the function of the remaining adrenal in both patients. These conclusions concur with those in the classical study of Liddle's group (2).

The CRH test is of no, or at the best, of minor value in discriminating patients with Cushing's syndrome from healthy subjects, as 30% of the patients with pituitary-dependent Cushing's disease have a normal cortisol response to CRH (Chapter 3.1). However, the CRH test is a reliable tool for subdividing patients with Cushing's syndrome into those with pituitary-

dependent Cushing's disease, ectopic ACTH secretion and adrenal-dependent Cushing's syndrome. In our study (Chapter 3.2) the diagnostic accuracy of the CRH test in the differential-diagnosis of Cushing's syndrome was at least as high as that of the "high-dose" dexamethasone suppression test according to Liddle, which is still considered the most reliable test for this purpose.

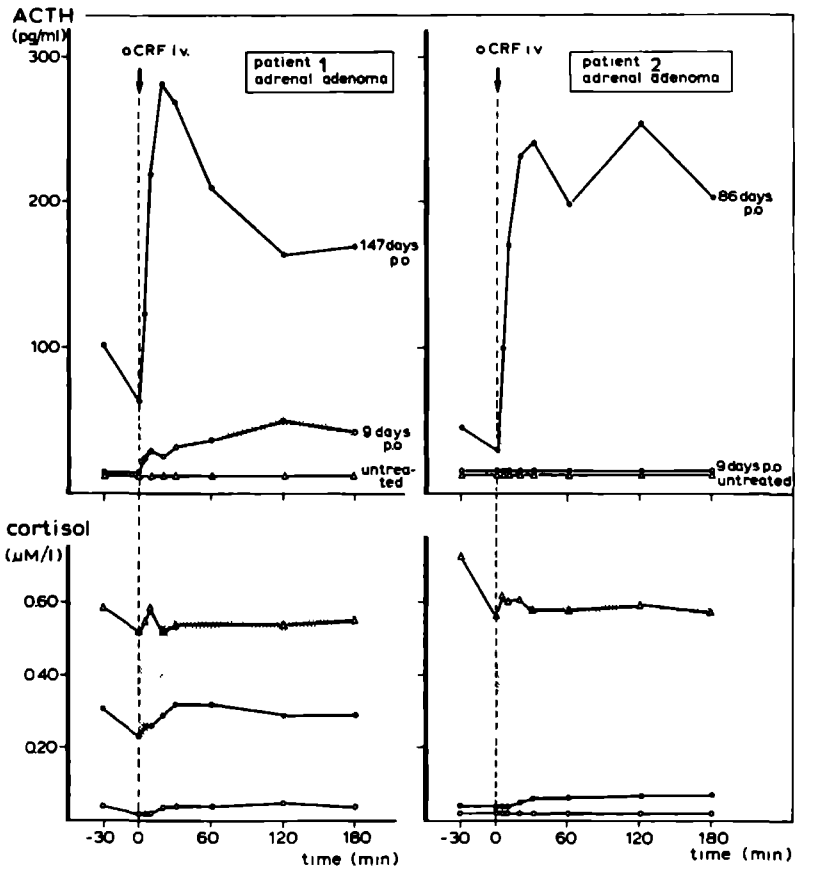

Figure 1 Plasma ACTH and cortisol responses to an intravenous bolus injection of 100 μg ovine CRF in two patients with Cushing's syndrome due to an adrenal adenoma (p.o.= postoperatively). The shaded area represents the mean ± 1SD in 13 normal subjects

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Samenvatting

In dit proefschrift worden effekten van de intraveneuze toediening van "Corticotropin-releasing hormone (CRH)" bij de mens beschreven. CRH is een hypothalaam hormoon dat de synthese en secretie van ACTH door de hypofyse bevordert. Het werd door Vale en medewerkers in 1981 geisoleerd uit hypothalami van schapen. In 1983 werd ook de structuur van het menselijke CRH opgehelderd door analyse van het pre-pro CRH gen van de mens. Deze beide CRH's zijn peptide-hormonen van 41 aminozuren, die niet geheel identiek zijn: op 7 plaatsen van de keten worden verschillende aminozuren gevonden. Immunologisch aantoonbare "CRH-activiteit" is niet beperkt tot de hypothalamus maar komt ook in een groot aantal andere delen van de hersenen voor. In Hoofdstuk 1 wordt onder andere de centrale rol van CRH bij de aanpassing van het organisme aan stress besproken.

In Hoofdstuk 2 worden gegevens vermeld die werden verkregen bij bestudering van de effekten van toediening van CRH aan gezonde volwassen mensen. In een eerste studie (Hoofdstuk 2.1) betreffen deze gegevens effekten van intraveneuze injectie, bij wijze van een "bolus", van 200 μg CRH van schapen, toegediend te 9 uur in de morgen aan 10 gezonde vrijwilligers. CRH-toediening leidde tot een, voor mannen en vrouwen niet verschillende, verdubbeling van de spiegels van ACTH, cortisol en aldosteron in plasma. Daarbij werd geen stijging waargenomen in de spiegels van gonadotrofinen, TSH, groeihormoon en prolactine. Er werd een zeer significante negatieve correlatie vastgesteld tussen de individuele basale spiegels van cortisol enerzijds en het antwoord van ACTH en cortisol na toediening van het CRH, gemeten aan het oppervlak onder de individuele ACTH- en cortisol-curven. De basale spiegels van ACTH evenwel waren niet gecorreleerd met de op deze wijze gemeten antwoorden van ACTH en cortisol op de toediening van CRH. Deze gegevens wijzen erop dat het antwoord van de hypofyse op CRH primair bepaald wordt door de

hoeveelheid in circulatie aanwezige glucocorticoide activiteit, dus door een op de hypofyse inwerkende factor en niet door de activiteit van de hypofyse met betrekking tot de ACTH produktie ten tijde van de toediening van het hormoon.

In Hoofdstuk 2.2 worden de effekten van bolus-injecties van 200 μg CRH van het schaap vergeleken met die van 200 μg CRH van de mens. Bij 10 gezonde proefpersonen bleek menselijk CRH een ongeveer gelijke stijging van de spiegels van ACTH en cortisol te bewerkstelligen als CRH van het schaap. Het verschil tussen beide was dat het menselijke CRH een significant kortere werkingsduur bleek te hebben. Bovendien werd waargenomen dat een dergelijke hoeveelheid humaan CRH, in tegenstelling tot het CRH van het schaap, de secretie van prolactine door de hypofyse deed toenemen.

Uit dierexperimenten was inmiddels gebleken dat intraveneuze toediening van CRH kan leiden tot een geringe daling van de bloeddruk, waarschijnlijk tengevolge van perifere vaatverwijding. In Hoofdstuk 2.3 worden hemodynamische effekten van intraveneuze toediening van CRH in een bolus bij 31 gezonde vrijwilligers geanalyseerd. Na injectie van 100 μg CRH van het schaap of de mens veranderde de bloeddruk niet. Na toediening van 200 μg CRH van het schaap daalde de diastolische bloeddruk gemiddeld 14 mmHg, terwijl dezelfde dosis CRH van de mens de diastolische bloeddruk significant meer omlaag bracht, gemiddeld 23 mmHg. Daling van de systolische bloeddruk trad in geen der experimenten op, ook niet na toediening van 200 μg CRH van de mens. Deze experimenten werden niet dubbelblind uitgevoerd en ook het effekt van toediening van het oplosmiddel zonder CRH werd niet systematisch bestudeerd. Toch menen wij dat, gezien de sterkere daling van de diastolische bloeddruk na toediening van menselijk CRH vergeleken met die na toediening van CRH van het schaap, voorlopig de voorkeur gegeven moet worden aan CRH van het schaap bij gebruik voor diagnostische doeleinden in de kliniek.

De hypothese dat het antwoord van de hypofyse-bijnieras op de toediening van CRH bij de mens wordt bepaald door de in circulatie aanwezige hoeveelheid cortisol, kreeg verder steun door experimenten beschreven in Hoofdstuk 2.4. Hierin wordt het antwoord van ACTH en cortisol op de toediening van 200 μg schape-CRH bij 5 gezonde vrijwilligers bestudeerd nadat deze met verschillende doses van het synthetische glucocorticoid dexamethason waren behandeld. Dit glucocorticoid werd in de avond voorafgaande aan de morgen van de CRH proef toegediend in zodanige hoeveelheden dat ten tijde van de injectie van CRH de spiegels van ACTH en cortisol in het bloed nagenoeg volledig gesupprimeerd waren. De concentratie van dexamethason in het plasma onmiddellijk voor toediening van CRH bleek negatief gecorreleerd te zijn met de grootte van het oppervlak onder de curven van ACTH en cortisol na toediening van CRH.

In Hoofdstuk 2.5 worden waarnemingen beschreven die eveneens een

sterk argument vormen voor de opvatting, dat het de concentratie van cortisol is, die het antwoord van de hypofyse op CRH in hoge mate bepaalt. Hierbij werden proefpersonen voorbehandeld met het antiglucocorticoid RU-486. Deze stof bezet receptoren voor cortisol en schakelt daardoor effekten van glucocorticoiden op doelwitorganen uit. Indien men aanneemt, dat deze stof de receptoren voor cortisol in de hypofyse blokkeert, en daarmee terugkoppeling van cortisol op de hypofyse uitschakelt, verwacht men dat de door CRH geinduceerde toeneming van ACTH-secretie wordt versterkt. Dit werd inderdaad vastgesteld. Bovendien wezen deze proeven uit dat toediening van 100 mg van het bovengenoemde antiglucocorticoid om 2 uur 's nachts de gemiddelde ACTH- en cortisol-spiegels in het bloed 6 tot 7 uur later, dus nog voor toediening van CRH, deed toenemen. Deze waarneming suggereert dat het antiglucocorticoid de receptoren voor cortisol in de hypofyse en/of de hypothalamus blokkeert. Daardoor kan circulerend cortisol de ACTH-secretie niet remmen en ontstaat de combinatie van verhoogde ACTH-spiegels met daarbij, en daardoor geinduceerd, verhoogde spiegels van cortisol in het bloed.

Na deze waarnemingen bij gezonde proefpersonen werd geanalyseerd welke factor het antwoord van ACTH op toediening van CRH bepaalt bij patienten bij wie de terugkoppeling door cortisol niet kan optreden, omdat cortisol in de circulatie nagenoeg ontbreekt - patienten met de ziekte van Addison - en bij patienten met de zogenaamde hypofyse-afhankelijke vorm van het syndroom van Cushing - een aandoening die wordt gekenmerkt door een primair defekt in de "feedback" van cortisol. Bij beide ziektebeelden bleek het absolute antwoord van ACTH op toediening van CRH van het schaap verhoogd te zijn. Dit antwoord was bovendien positief gecorreleerd met de basale spiegel van ACTH, terwijl geen significante relatie kon worden aangetoond tussen de basale spiegel van circulerend cortisol en het antwoord van de hypofyse op CRH (Hoofdstuk 3.1 en 3.3). De resultaten van de onderzoekingen bij patienten met de ziekte van Addison suggereren het volgende: door het vrijwel ontbreken van cortisol in de circulatie kan van feedback door cortisol natuurlijk geen sprake zijn en in die situatie komt blijkbaar aan de basale activiteit van de hypofyse qua produktie van ACTH een regulerende rol toe bij het antwoord op CRH. Bij patienten met de ziekte van Cushing verklaren wij het gevonden resultaat als een rechtstreeks gevolg van een primair defekt in de receptie van circulerend cortisol door de corticotrope cellen. Bij deze laatste groep van patienten vonden wij ook een dergelijke positieve correlatie tussen de ACTH-spiegel na suppressie met hogere en lagere doseringen van het synthetische glucocorticoid dexamethason en het antwoord van de hypofyse op CRH (Hoofdstuk 3.1).

Het syndroom van Cushing wordt veroorzaakt door primaire overproduktie van ACTH, hetzij door de hypofyse hetzij door een ectopische bron, of door

primaire overproduktie van cortisol door een bijnieradenoom of -carcinoom. Om deze verschillende vormen van het syndroom van Cushing te onderscheiden zijn verschillende diagnostische onderzoekingen in gebruik. Nogal eens leiden deze proeven tot resultaten die met elkaar in strijd zijn. Na de isolatie van CRH hebben verschillende groepen onderzoekers de waarde van CRH voor de differentiele diagnostiek van het syndroom van Cushing onderzocht. De overigens nog schaarse literatuurgegevens laten zien dat patienten met een hypofyse-afhankelijke vorm van het syndroom van Cushing meestal een stijging van de plasma-spiegels van ACTH na toediening van CRH laten zien, terwijl patienten met een bijnier-afhankelijke vorm gekenmerkt worden door niet detecteerbaar lage spiegels van ACTH basaal, die na toediening van CRH niet stijgen. Patienten met de ectopische vorm van het syndroom van Cushing hebben meestal sterk verhoogde basale spiegels van ACTH, die niet verder stijgen na CRH-injectie.

Wij vergeleken de waarde van de CRH test (toediening van 100 μg schape-CRH als bolus om 9 uur 's ochtends) met die van de orale "hoge dosis" dexamethason suppressietest volgens Liddle voor de differentiele diagnostiek van het syndroom van Cushing bij 26 achtereenvolgens bestudeerde patienten met dit ziektebeeld (Hoofdstuk 3.2). Een vals-negatief antwoord na CRH kwam voor bij 2 van 22 (9%) patienten met de hypofyse-afhankelijke vorm van dit ziektebeeld, terwijl een dergelijk fout antwoord werd gevonden bij 2 van 18 (11%) van deze patienten bij gebruik van de Liddle test. Drie patienten met het syndroom van Cushing ten gevolge van een bewezen bijnieradenoom reageerden volgens verwachting noch op CRH noch op toediening van dexamethason. Eén patient met de ectopische vorm van het syndroom van Cushing toonde een vals-positieve reactie van ACTH na toediening van dexamethason maar geen antwoord op CRH. Een fout antwoord van beide testen kwam bij geen van de onderzochte patienten voor, met uitzondering van één patient met de ziekte van Cushing en een uitgesproken macronodulaire hyperplasie van de bijnieren. Overigens moet hieraan worden toegevoegd dat het bij deze interessante vorm van het syndroom van Cushing de vraag is welk antwoord men op elk van beide testen theoretisch zou moeten verwachten. Uit deze studie werd geconcludeerd dat de diagnostische waarde van de CRH test bij de differentiele diagnostiek van het syndroom van Cushing goed vergelijkbaar is met het resultaat van de Liddle test, tot op heden nog steeds beschouwd als de meest betrouwbare proef bij deze vorm van differentiele diagnostiek. Bij combinatie van de resultaten van beide proeven bereikt men een nagenoeg 100% score. Daarbij moet wel duidelijk onderstreept worden dat gebruik van de CRH test om poliklinische patienten zonder het syndroom van Cushing te onderscheiden van patienten met dit syndroom van weinig waarde is. Immers bij 30% van de patienten met een hypofyse-afhankelijke vorm van

het syndroom van Cushing bleek het antwoord van cortisol op toediening van CRH niet te onderscheiden van dat bij gezonde vrijwilligers.

In Hoofdstuk 3.3 werd aangetoond dat, door bestudering van het antwoord op CRH, patienten met secundaire bijnierschorsinsufficientie in twee groepen kunnen worden onderscheiden: patienten die geen antwoord van ACTH op toediening van CRH laten zien en patienten met wel een antwoord van ACTH na toediening van CRH. Deze laatstgenoemde patienten bleken vaak een bifasische ACTH respons te vertonen, terwijl de gemiddelde ACTH respons na CRH bij deze patienten significant hoger was dan bij gezonde proefpersonen. Ten aanzien van deze laatste groep van patienten mag in alle redelijkheid gesteld worden dat bij hen de oorzaak van de bijnierschorsinsufficientie niet gelegen is in de hypofyse, maar in de hypothalamus of op een nog hoger niveau. Het spreekt veel minder vanzelf dat de oorzaak van de bijnierschorsinsufficientie bij de eerstgenoemde groep van patienten in de hypofyse moet worden gezocht. Het is immers bekend dat bij zogenaamde hypothalame insufficientie het antwoord van de hypofyse op andere "releasing"-hormonen (TRH, LHRH, groeihormoon-releasing hormoon) soms pas op gang komt na herhaalde stimulering. Een aantal klinische en biochemische gegevens, die in Hoofdstuk 3.3 zijn vermeld, maken het overigens waarschijnlijk dat de oorzaak van de bijnierschorsinsufficientie van deze eerstgenoemde groep toch in de hypofyse moet worden gezocht. Hoewel anatomische bewijsvoering voor deze stellingname ontbreekt, menen wij met enige voorzichtigheid toch te mogen concluderen dat de CRH test nuttig gebruikt kan worden om bij patienten met secundaire bijnierschorsinsufficientie het niveau van de lesie te localiseren.

Patienten met de ziekte van Cushing, bij wie door middel van een neurochirurgische ingreep de in de hypofyse zetelende oorzaak is weggenomen, blijken na de operatie tijdelijk een onvoldoende bijnierschorsfunctie te hebben. Deze bijnierschorsinsufficientie is van het zogenaamde "secundaire" type. Het is niet bekend of dit beeld veroorzaakt wordt door onvoldoende functie van het resterende deel van de hypofyse of het gevolg is van onvoldoende stimulering van de ACTH-produktie vanuit de hypothalamus. In Hoofdstuk 3.4 bestudeerden wij door middel van CRH-toediening het functioneren van de hypofyse en de bijnierschors bij 8 patienten met de ziekte van Cushing, korte tijd (9 tot 18 dagen) na, blijkens het verdere beloop, geslaagde chirurgie van de hypofyse. Deze 8 patienten waren allen na de operatie hypocortisolemisch. Niettemin leidde toediening van CRH bij 7 van deze patienten tot een duidelijke toeneming van de spiegels van ACTH en cortisol in bloed. Deze waarneming moet er op duiden dat er na deze vorm van hypofyse-chirurgie blijkbaar sprake was van onvoldoende stimulering van het resterende deel van de hypofyse vanuit de hypothalamus. Op zich pleit deze waarneming tegen een oorzakelijke rol van

CRH bij het ontstaan van de ziekte van Cushing. Bij vergelijking van deze serie resultaten met die verkregen bij een groep patienten met onvoldoende bijnierschorsfunctie van het hypothalame type, zoals beschreven in Hoofdstuk 3.3, bleek dat de gemiddelde toeneming van de spiegels van ACTH in het bloed van de 8 patienten na neurochirurgie wegens de ziekte van Cushing significant lager was dan de gemiddelde toeneming bij de eerstgenoemde groep patienten. Het komt ons daarom voor dat patienten met de ziekte van Cushing na operatie niet alleen een onvoldoende bijnierschorsfunctie hebben als gevolg van een gebrekkige sturing van de ACTH-secretie vanuit de hypothalamus maar tevens een zogenaamde "hypofysaire" insufficientie. Deze combinatie van gestoorde functie van hypothalamus en hypofyse na verwijdering van een hypofyse-adenoom wordt waarschijnlijk veroorzaakt door suppressie van zowel de cellen in de hypothalamus die CRH produceren als van de resterende hypofyse-cellen, betrokken bij de secretie van ACTH. Deze combinatie van afwijkingen wordt waarschijnlijk veroorzaakt door de in het verleden langdurig verhoogde spiegels van circulerend cortisol in bloed.

In Hoofdstuk 4 worden een aantal onverwachte reacties beschreven, die optraden bij enkele patienten na toediening van CRH. Het is mogelijk dat enige van deze reacties (kortdurende absences en dalingen van de bloeddruk) veroorzaakt werden door toediening van een te grote hoeveelheid van het peptide. Twee andere reacties traden waarschijnlijk op zonder een directe relatie met de toediening van CRH. Bij één patient kwam de combinatie van bloeddrukdaling en bradycardie voor, die mogelijk werd veroorzaakt door een, vooralsnog niet bewezen, interactie tussen CRH en het heparine, dat gebruikt werd om de cannule voor afname van veneus bloed open te houden. In dit hoofdstuk wordt er op gewezen dat de studie naar de oorzaak van deze onverwachte reactie nog wordt voortgezet.

Dankwoord

Het is mij een genoegen de velen, die aan de totstandkoming van dit proefschrift een belangrijke bijdrage geleverd hebben, te kunnen bedanken. Dit geldt op de eerste plaats voor de patienten en gezonde vrijwilligers, die aan dit onderzoek meewerkten.

De onderzoeken bij de klinische patienten werden uitgevoerd door de arts-assistenten en verpleegkundigen van de afdeling C-10.
De deskundige leiding van Zr. C. Willemsen en Zr. T. Terburg zorgde ervoor dat er nooit een test "de mist in ging". De sfeer op de Dagverpleging (hoofd: Zr. L. Schreppers) van de Polikliniek voor Inwendige Ziekten was zodanig dat de gezonde proefpersonen hier steeds graag terugkwamen.

Dit onderzoek kwam tot stand in nauwe samenwerking met de afdeling Experimentele en Chemische Endocrinologie (hoofd: Prof. Dr. Th.J. Benraad). De bloedmonsters werden steeds nauwkeurig verwerkt door Mevr. H. Boogerd. De vele hormoonbepalingen werden uitgevoerd door Mevr. B. Theelen, Mevr. A. van Geel, de Heer J. Willemsen, de Heer J. Hofman, de Heer M. Barten en de Heer H. Coenen. Zeer veel dank ben ik verschuldigd aan de Heer G. Pesman, die niet alleen op nauwkeurige wijze de ACTH bepalingen uitvoerde, doch ook vaak een belangrijke schakel was tussen laboratorium. Bovendien was hij steeds actief betrokken bij de opzet van het onderzoek.

Mevr. Drs. W. Buijs en Dr. H.A. Ross adviseerden bij de statistische verwerking van de gegevens. Mevr. W. Straten, Mevr. I. Swinkels-Scholten en Mevr. W. Janssen-Bruijns hebben met veel ijver de verschillende versies van de hoofdstukken van dit proefschrift getypt. Mevr. W. Straten heeft bovendien met niet aflatend enthousiasme de definitieve versie verzorgd.

Dank ook aan de medewerkers van de Medische Bibliotheek (destijds hoofd: de Heer E. de Graaff, huidig hoofd: Mevr. Drs. S. Bakker) en van de afdeling Medische Fotografie (hoofd: de Heer A. Reijnen). De Heer J. Konings, de Heer J. Wiese en wijlen de Heer H. Berris illustreerden dit

proefschrift op vakkundige wijze.

Drs. J.H. Bakker van de afdeling Klinische Farmacie was nauw betrokken bij het onderzoek naar de bijwerkingen van CRH toediening. Dit onderzoek kon slechts uitgevoerd worden dankzij de inspanningen van Dr. G.P. Chrousos (National Institutes of Health, Bethesda, U.S.A.), Prof. Dr. C.W. Hilbers en de Heer J. Joordens (afdeling Biofysische Chemie), Dr. J. Smits en Mevr. H. van Essen (capaciteitsgroep Farmacologie, Universiteit van Maastricht) en de Heer T. Arts (Centraal Dieren Laboratorium, hoofd: Dr. W. van der Gulden).

Tenslotte hecht ik eraan mijn ouders te bedankenn voor de mogelijkheden die ze mij geboden hebben en voor hun geduld, wanneer ik weer eens geen tijd had.

Curriculum Vitae

De schrijver van dit proefschrift werd in 1955 geboren. Hij behaalde in 1973 het diploma gymnasium-β aan het Sint Norbertuslyceum te Roosendaal. In hetzelfde jaar begon hij met zijn studie in de geneeskunde aan de Katholieke Universiteit te Nijmegen, waar hij in 1978 het doctoraal examen en in 1980 het artsexamen aflegde. Daarna begon hij zijn opleiding tot internist in het Groot Ziekengasthuis te 's-Hertogenbosch (opleider: Dr.J.B.Lips). Deze opleiding werd in 1982 voortgezet aan de Universiteitskliniek voor Inwendige Ziekten van het Sint Radboudziekenhuis te Nijmegen (opleider: Prof.Dr.A.van 't Laar). De registratie tot internist vond plaats in augustus 1985. Sinds 1 januari 1986 is hij werkzaam op de afdeling Endocriene Ziekten (hoofd: Prof.Dr.P.W.C.Kloppenborg) van de Universiteitskliniek voor Inwendige Ziekten te Nijmegen.

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Stellingen

1 Bij gezonde volwassenen wordt het antwoord van ACTH na toediening van CRH bepaald door de hoeveelheid glucocorticoide aktiviteit, die in de circulatie aanwezig is: hoe lager de basale cortisol concentratie, des te sterker het antwoord van ACTH na CRH toediening.

Dit proefschrift

2 Bij patiënten met de ziekte van Cushing of de ziekte van Addison wordt het antwoord van ACTH na toediening van CRH niet bepaald door de hoeveelheid glucocorticoide aktiviteit, die in de circulatie aanwezig is, doch door de basale aktiviteit van de hypofyse qua produktie van ACTH: hoe hoger de basale ACTH concentratie, des te sterker het antwoord van ACTH na CRH toediening.

Dit proefschrift

3 De diagnostische waarde van de CRH test bij de differentiële diagnostiek van het syndroom van Cushing is vergelijkbaar met die van de klassieke dexamethason suppressietest volgens Liddle.

Dit proefschrift

4 De CRH test kan bij patiënten met secundaire bijnierschorsinsufficiëntie differentiëren tussen hypothalame en hypofysaire oorzaken van dit ziektebeeld.

Dit proefschrift

5 Bij patiënten met hypertensie en hypokaliemie dient ook het bestaan van een renine-producerende tumor overwogen te worden.

A. Hermus, G. Pieters, A. Lamers et al. Hypertension and hypokalemia due to a renin-secreting kidney tumour. Neth. J. Med. 1986, in press.

6 Voor het vroegtijdig opsporen van metastasen bij patiënten, eerder behandeld wegens een primair mammacarcinoom is gericht onderzoek naar aanleiding van een bepaalde klacht nog steeds effektiever dan routinematige "screening" middels scans en röntgenfoto's.

7 Een tekort aan CRH in de hersenschors speelt mogelijk een rol bij de ontwikkeling van preseniele dementie van het type Alzheimer.

E. De Souza, P. Whitehouse, M. Kuhar et al. Reciprocal changes in corticotropin-releasing factor (CRF)-like immunoreactivity and CRF receptors in cerebral cortex of Alzheimer's disease. Nature (1986), 319, 593.

8 Het verdient nader onderzoek of produktie van CRH en van ACTH door de placenta een rol speelt bij de ontwikkeling van het fysiologische hypercorticisme in de tweede helft van de zwangerschap.

9 Bij langdurig ernstig zieke patiënten moet men bij onbegrepen hypotensie, die onvoldoende reageert op toediening van pressor-aminen bedacht zijn op het bestaan van een relatieve bijnierschorsinsufficientie. Het vinden van een "normale" cortisolspiegel sluit deze diagnose onder dergelijke omstandigheden niet uit. Toediening van glucocorticoiden in voldoende hoge dosering kan bij deze patiënten de circulatoire stabiliteit aanzienlijk vergroten.

Recente waarnemingen

10 Het doorbrengen van een gedeelte van de opleidingstijd tot medisch specialist in een "perifeer" ziekenhuis is niet alleen opleidingstechnisch gewenst, doch voorkomt ook de in sommige academische kringen heersende vooroordelen over "het ziekenhuis elders".

11 De vraag om bij ernstig zieke patiënten een medische behandeling te staken is veel vaker van familieleden afkomstig dan van de patiënt zelf.

12 De definitie van Gezondheid, zoals vastgesteld door de World Health Organization: "Een toestand van volkomen biologisch, sociaal en psychisch welbevinden", is utopisch. Aldus gedefiniëerd zal Gezondheid slechts beleefd worden in Utopia, in Nergensland dus. Een dergelijke voorstelling van Gezondheid wekt van de Gezondheidszorg verwachtingen, die niet te vervullen zijn.

Nijmegen, 17 april 1986

Printed by ICG Printing, Dordrecht